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TITLE: Optically Based Rapid Screening Method for Proven Optimal Treatment Strategies before Treatment Begins

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| 14. ABSTRACT This award seeks to develop high-throughput optical metabolic imaging (OMI) of primary breast tumor organoids (three-dimensional cultures of tumors) to rapidly test/screen breast cancer therapeutics as a strategy to streamline drug development and provide individualized treatment. The results outlined in this progress report indicate that OMI in primary breast tumor organoids can provide early, sensitive measures of treatment response. This system can therefore be used to streamline pre-clinical drug development, by reducing the number of animals, cost, and time required to screen new drugs and drug combinations. The feasibility of OMI measurements in organoids derived from primary human breast tumors has also been demonstrated, with the exciting possibility of providing individualized drug screens in patients before the patient is ever treated. This personalized drug planning approach could reduce toxicities of ineffective treatments, and enable improved outcomes through early, effective interventions. The single-cell analysis capabilities of this technique also have the potential to identify drug combinations that will eliminate treatment-resistant sub-populations of cells that are likely to cause metastases. | | | | | |
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INTRODUCTION

This award seeks to develop high-throughput optical metabolic imaging (OMI) of primary breast tumor organoids (three-dimensional cultures of tumors) to rapidly test/screen breast cancer therapeutics as a strategy to streamline drug development and provide individualized treatment. This award tests the hypothesis that early changes in tumor cell metabolism as measured by OMI can be used to predict breast tumor response to multiple treatment conditions. The award has three aims. The first is to determine the earliest time point when therapy-induced changes in OMI can be detected. The second is to measure dose-dependent changes in metabolism in response to chemotherapeutic drugs. The third is to compare the results from OMI in patient tissue organoids to actual patient response determined from standard-of-care.

BODY

Aim 1: Determine the earliest time-point at which therapeutically-induced metabolic changes can be measured. (Months 1-24).

1a. Regulatory review and approval for animal studies (months 1-2).
Completed.

1b. Measure mouse organoid response to drug treatments alone and in combination over a time course with n=480 samples from 9 mice using optical metabolic imaging (OMI) (months 3-15).

Completed. The details of this work are described in a manuscript that was just accepted to the journal Cancer Research¹. Briefly, we have successfully established the organoid culture system from human breast tumor xenografts grown orthotopically in nude mice (*Figs. 1-2*). The feasibility of the organoid-OMI approach for high-throughput drug screening has also been confirmed in xenografts that are responsive (*Fig. 1*) and resistant (*Fig. 2*) to trastuzumab. In the responsive tumors, long-term *in vivo* tumor volume measurements (*Fig. 1A-B*) corroborate the fast OMI read-outs (72hr) of response obtained for multiple drugs in organoid culture (*Fig. 1D*). Responsive BT474 tumors regressed with paclitaxel

(standard chemotherapy), trastuzumab, XL147 (PI3K-inhibitor), and combination treatments (*Fig. 1D*). This response was **resolved on a cellular level** (*Fig. 1C&E*), with a bi-modal response to paclitaxel and trastuzumab treatment, and a single population in control and combination-treatment groups. This single-cell analysis indicates that sub-populations of cells respond differently to drug treatment, and illustrates the power of cellular-level imaging for quantifying heterogeneous tumor response to treatment. OMI-measured response was corroborated with immunohistochemistry (IHC; *Fig. 1F*).

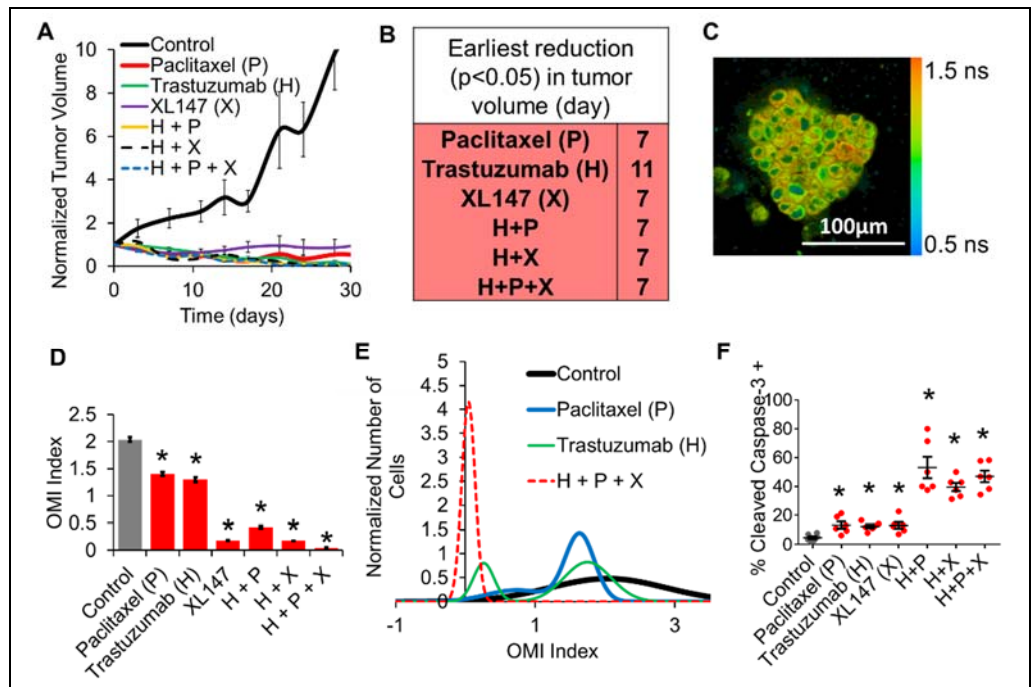
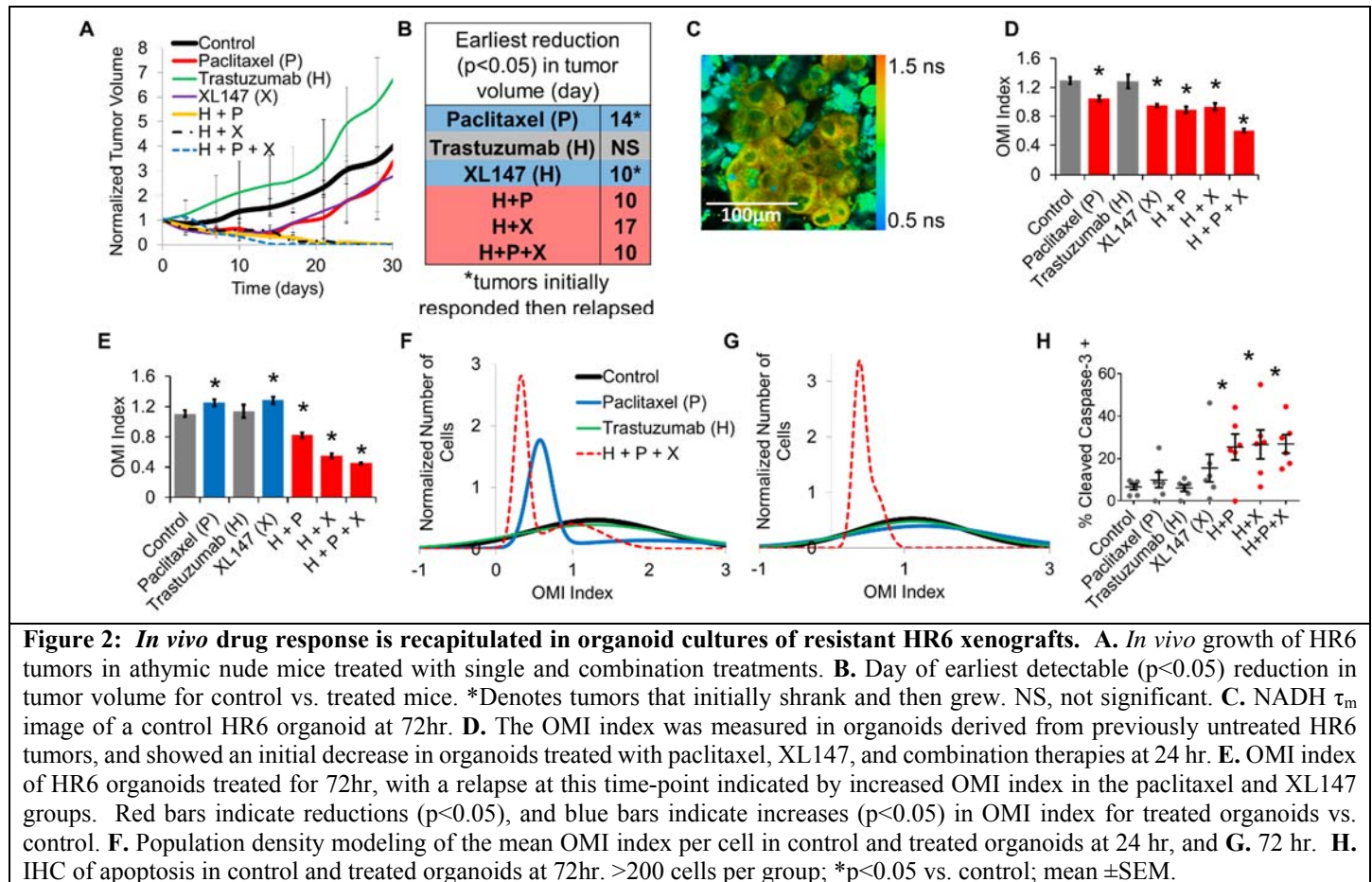


Figure 1: *In vivo* drug response is recapitulated in organoid cultures of responsive BT474 xenografts. **A.** *In vivo* growth of BT474 tumors grown in nude mice and treated with single and combination treatments. **B.** Day of earliest reduction ($p < 0.05$) in tumor volume for control vs. treated mice. **C.** NADH fluorescence lifetime (τ_m) image of a control BT474 organoid at 72hr. **D.** The OMI index in organoids derived from previously untreated BT474 tumors decrease with single and combination therapies at 72hr. **E.** Population density modeling of the mean OMI index per cell in control and treated organoids at 72hr. **F.** IHC of apoptosis in BT474 organoids at 72hr. >200 cells per group; * $p < 0.05$ vs. control.

In trastuzumab-resistant xenografts (**Fig. 2**), long-term tumor volume measurements confirmed that tumors continue to grow with trastuzumab-treatment, continue to shrink with combination treatments, and exhibit an initial response and then re-growth with paclitaxel and XL147 treatment (**Fig. 2A-B**). Remarkably, this *in vivo* drug response is recapitulated with OMI measurements at 24 and 72 hours post-treatment, in organoids derived from these previously untreated drug-resistant xenografts (**Fig. 2D-E**). After 24 hours, all treatment groups except trastuzumab show an initial drug response (**Fig. 2D**). However after 72 hours of treatment, the paclitaxel and XL147 groups have an increased OMI index (indicating non-response, **Fig. 2E**). All combination treatments and trastuzumab-treated organoids retain a response and lack of response, respectively, at both 24 and 72 hours (**Fig. 2D-E**). Therefore, OMI in tumor organoids accurately predicts response, resistance, and regression at early time-points (24 and 72 hours) compared to *in vivo* tumor volume (10-17 days). Cellular-level population modeling reveals longitudinal changes in cell drug response, with two sub-populations of paclitaxel-treated cells at 24 hours (**Fig. 2F**), and a single population of paclitaxel-treated cells at 72 hours that overlaps with control (**Fig. 2G**). In contrast, the combination treatment group has two sub-populations at 24 hours (**Fig. 2F**) that nearly merge into one responsive population at 72 hours (**Fig. 2G**). Response is confirmed with IHC (**Fig. 2H**).



This powerful data (**Fig. 1-2**) of 6 treatment groups in 2 xenograft models suggests that the organoid-OMI system provides an accurate model of *in vivo* tumor drug response with respect to *in vivo* gold standards (tumor volume). This high sensitivity to early drug response is due to cellular-level, functional images of metabolism that are early predictors of response. Importantly, the multi-drug OMI screen in organoids was achieved within 72h with only 1 mouse per cell line, whereas the tumor volume response required 7-17 days and 21 mice per cell line to complete. Therefore the OMI-organoid platform provides a 2 to 5 times reduction in readout time and at least a 21X reduction in mouse burden for drug testing. We have done as many as 30 drug screens from one mouse tumor with the organoid system, so the potential reduction in drug screening costs with

this system could be much higher. Additionally, OMI measurements allow for cell-to-cell heterogeneity in drug response to be quantified (**Fig. 1E**, **Fig. 2F-G**), to refine therapies to induce response in all cell sub-populations.

- 1c. Measure *in vivo* response to drug treatments alone and in combination over a time course with n=270 mice using OMI (months 3-24).

Completed. The details of this work are described in a manuscript published in Cancer Research². Briefly, our published data² indicate that OMI is sensitive to treatment-induced changes in cellular metabolism *in vivo* at earlier time-points (48 hours) than any reported biomarker of trastuzumab-response *in vivo* (**Fig. 3**), further supporting OMI development. The “OMI index” is a linear combination of the redox ratio, mean NADH lifetime, and mean FAD lifetime (OMI index = redox ratio + NADH τ_m – FAD τ_m). The OMI index was developed to provide the most accurate combination of variables to measure drug response while maximizing differences between control/non-responsive and drug responsive groups. A decrease in the OMI index compared to control indicates response to treatment (i.e. decreased glycolysis and protein-binding³), and an increase / no change in the OMI index indicates non-response. These OMI-resolved metabolic changes (**Fig. 3B**) precede changes in tumor volume by 6 days (**Fig. 3C**), agree with invasive measures of proliferation (**Fig. 1D**), and improve upon current *in vivo* metabolic measures of response (FDG-PET) that showed no change within the 14-day time course (**Fig. 3E**)². Furthermore, distribution modeling of the *in vivo* OMI index of MDA-MB-361 xenografts (**Fig. 3F**), which have a known heterogeneous response to trastuzumab⁴, identifies two distinct cell sub-populations that arise in response to trastuzumab treatment. The relative weights of these two cell sub-populations (30%/70% for low/high redox ratio distributions) is in agreement with the 25% of MDA-MB-361 cells that are responsive to trastuzumab *in vitro*⁴. This suggests that OMI is capable of resolving a heterogeneous cellular drug response *in vivo*. Conversely, *in vivo* OMI (**Fig. 3G**) and tumor volume measurements (**Fig. 3H**) from trastuzumab-resistant HR6 tumors show no changes between control and trastuzumab-treated tumors at any time point within 14 days.

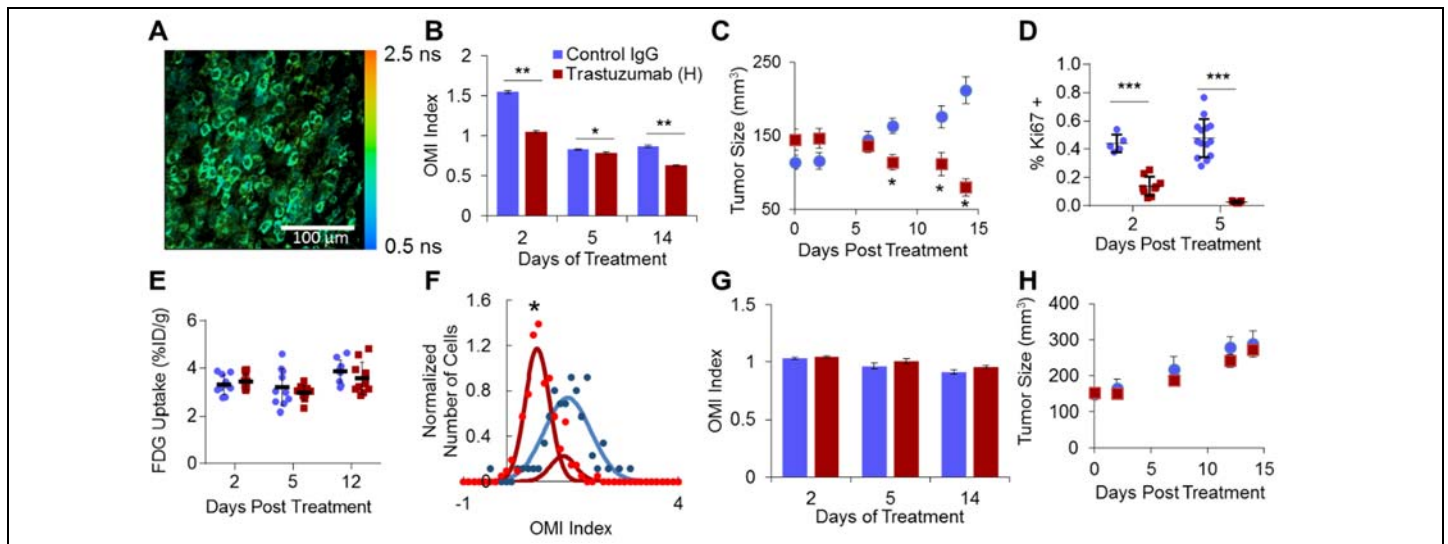


Figure 3: *In vivo* OMI resolves response to trastuzumab treatment². (A) Representative *in vivo* NADH τ_m image from a BT474 (ER+/HER2+) xenograft. (B) Significant decreases in OMI index are observed 48h after one drug treatment (25 mg/kg trastuzumab every 3d) in responsive BT474 tumors. (C) Tumor size in BT474 tumors. (D) Proliferation in BT474 tumors. (E) No significant differences in FDG uptake are observed at any time point up to day 12 in BT474 tumors. (F) Cellular distribution density modeling of the *in vivo* OMI index in control (blue line, uni-modal) and trastuzumab-treated (maroon, bi-modal) MDA-MB-361 tumors at 48h, which have a heterogeneous response to trastuzumab⁴. Trastuzumab-resistant HER2+ HR6 tumors show no change in the *in vivo* (G) OMI index, or (H) tumor size between control and trastuzumab-treated mice. *p<0.05, **p<0.01, ***p<0.001 trastuzumab vs. control.

- 1d. Analyze OMI data collected from organoid and *in vivo* time-course experiments (months 3-24). Completed. See **Figs. 1-3**, and publications.

- 1e. Perform immunohistochemistry (IHC) analysis on organoids (from 1b) and tumors imaged *in vivo* (from 1c) (months 3-24).

Completed. See **Figs. 1-3**, and publications.

Milestone #1: These experiments will validate our organoid-OMI methods relative to *in vivo* measures and gold standards (IHC) with respect to *temporal* response. These experiments will also guide the selection of time-points for use in future studies.

Aim 2: Measure dose-dependent metabolic changes due to clinically-relevant breast cancer drugs. (Months 13-36).

2a. Measure mouse organoid response to drug treatments alone and in combination over a dosage range with n=540 samples from 9 mice using OMI (months 13-24).

This task is currently underway.

2b. Measure *in vivo* response to drug treatments alone and in combination over a time course with n=200 mice using OMI (months 13-33).

This task is currently underway.

2c. Analyze OMI data collected from organoid and *in vivo* dose-response experiments (months 13-36).

This task is currently underway.

2d. Perform IHC analysis on organoids (from 2a) and tumors imaged *in vivo* (from 2b) (months 13-36).

This task is currently underway.

Milestone #2: This aim will validate our organoid-OMI methods relative to *in vivo* measures and gold standards (IHC) with respect to *dose* response. This work will set the stage for future dose-response studies of breast cancer therapeutics, potentially informing on the optimal drug concentrations for use in patients.

Aim 3: Compare the results of OMI-measured response in human tissue organoids with current clinical measures of response (Months 1-36).

3a. Regulatory review and approval for human studies (months 1-2).

Completed. IRB# BRE140263 and BRE03103 at Vanderbilt.

3b. Measure human organoid response to drug treatments alone and in combination from n=50 human breast tumor surgical specimens using OMI (months 3-36). Quarterly enrollment target of 4-5 patients.

We have completed measurements in 10 patients so far, 6 of which are published in the manuscript that was just accepted at Cancer Research¹. Briefly, we have optimized our organoid culture preparation on primary human breast tumor samples (**Fig. 4**). Fresh surgical specimens are immediately placed in chilled DMEM media and processed into organoids within 4h of excision. Our published studies⁵ demonstrate that OMI endpoints do not significantly change (<10%) relative to *in vivo* values when tissues are maintained in chilled DMEM for up to 12h after surgical excision. Therefore, we have a realistic timeframe to transport tissues to our lab. We have established that:

- 1) We can consistently generate organoids from primary human breast tumors, across 10 patients so far.
- 2) OMI of untreated primary-tumor derived organoids differentiates the three breast cancer sub-types: triple negative, ER+, and HER2+ (**Fig. 4B**). This trend is consistent with OMI in immortalized human cell lines².
- 3) Sufficient replicates (n=6 organoids/treatment) can be generated from a small (<1mm diameter) piece of primary human breast tumor to achieve statistically significant changes (p<0.05) between control and treated samples just 72h after drug treatment (**Fig. 4C,F,H**).

- 4) Changes in the OMI index (**Fig. 4C**) agree with IHC of apoptosis obtained with destructive techniques (**Fig. 4D**), in organoids derived from the same patient.
- 5) Treatment-induced changes in the OMI index are resolved in response to chemotherapy and targeted therapy in ER+/HER2- (**Fig. 4C&F**), ER-/HER2+ (**Fig. 4H**), and triple negative (not shown) patients.
- 6) Cellular-level assessments (**Fig. 4E,G**) resolve sub-populations of varying response in these heterogeneous human tumor organoids for combination treatments (**Fig. 4E** red and blue, **Fig. 4G** yellow) and non-responsive groups (**Fig. 4G**, purple).

Together, these data indicate that our methods of tissue collection, organoid generation, and OMI measures of drug response are feasible in primary human breast tumor samples on the size scale of core biopsy samples.

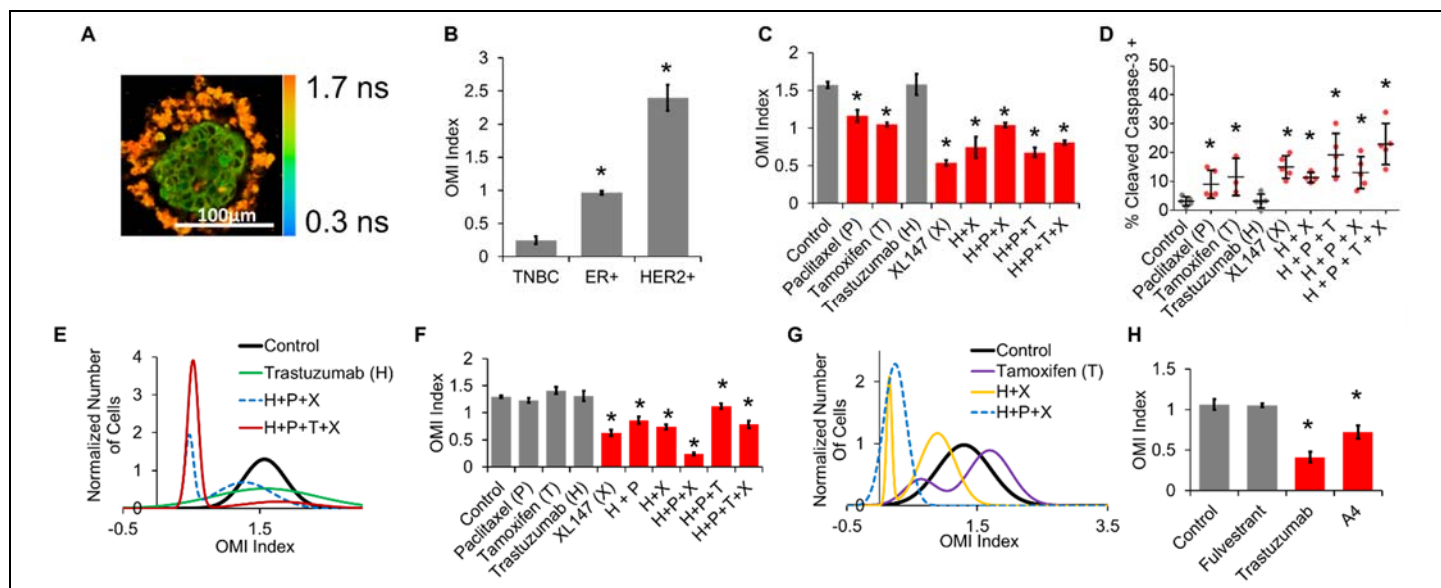


Figure 4: Feasibility of phenotyping and drug screening in organoids derived from fresh human tumor samples that have not been previously treated. (A) FLIM of FAD τ_m from a primary human tumor organoid. (B) OMI index of organoids derived from triple negative, ER+, and HER2+ primary human tumors not treated with any drugs show that OMI endpoints differ among breast cancer subtypes. (C) Multi-drug tests in organoids generated from an ER+/HER2- patient 72h post-treatment, with (D) corroborating stains for apoptosis in organoids generated from the same patient. (E) Population density modeling of a sub-set of data presented in (C). Multi-drug tests in organoids generated from (F) another ER+/HER2- patient, with (G) corresponding population density modeling, and (H) an ER-/HER2+ patient. Drug treatments include paclitaxel (P), tamoxifen (T, an ER-inhibitor), trastuzumab (H, HER2-inhibitor), XL147 (X, PI3K-inhibitor), fulvestrant (ER-inhibitor), and A4 (ErbB3-inhibitor). Note that different drugs were tested as methods were optimized in these pilot samples. >200 cells per group; * $p < 0.05$ vs. control, indicated by red bars/dots.

- 3c. Collect and analyze surgical pathology and FDG-PET results from patients recruited for this study (months 3-36).

This task is currently underway.

- 3d. Analyze OMI data collected from human organoid experiments (months 3-36).

This task is currently underway, see **Fig. 4** and published results.

- 3e. Perform IHC analysis on human organoid tissues (months 3-36).

This task is currently underway, see **Fig. 4** and published results.

Milestone #3: The results from this aim will assess the clinical utility of the organoid-OMI approach, and its accuracy with respect to gold standards (surgical pathology) and standard measures of response (FDG-PET). Ultimately, this work could provide a feasible path for high-throughput assessment of optimal treatment methods for breast cancer patients before treatment begins, significantly reducing morbidity and mortality.

KEY RESEARCH ACCOMPLISHMENTS:

- OMI measurements in organoids can resolve multi-drug response within 24-72 hours of treatment that are in agreement with gold standards of *in vivo* response (tumor volume, IHC).
- It is feasible to grow primary human tumor organoids from fresh breast tumor specimens.
- OMI measurements in primary human tumor organoids agree with standard IHC measures of response.
- OMI can resolve response on a cellular level, in both mouse xenografts and human tumors.

REPORTABLE OUTCOMES

Manuscripts:

1. AJ Walsh, RS Cook, CL Arteaga, MC Skala. Optical metabolic imaging of live tissue cultures. SPIE Proceedings. 2013; 8588.
2. AJ Walsh, RS Cook, HC Manning, DJ Hicks, A Lafontant, CL Arteaga, and MC Skala. Optical metabolic imaging identifies breast cancer glycolytic levels, sub-types, and early treatment response. Cancer Research (2013) Oct 15;73(20):6164-74.
3. AJ Walsh, MC Skala. An automated image processing routine for segmentation of cell cytoplasm in high-resolution autofluorescence images. SPIE Proceedings. 2014;8948.
4. AJ Walsh, RS Cook, ME Sanders, L Aurisicchio, G Ciliberto, CL Arteaga, and MC Skala. Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer. Cancer Research (2014), accepted.

Presentations:

1. AJ Walsh, RS Cook, ME Sanders, CL Arteaga, MC Skala, "Optical metabolic imaging predicts drug response in primary-tumor derived organoids." Gordon Research Conference: Lasers in Medicine and Biology. July 2014. Poster Presentation. **Poster Award.**
2. MC Skala, AJ Walsh, RS Cook, ME Sanders, CL Arteaga. "Cellular-Level Metabolic Imaging to Predict Anti-Cancer Drug Response." Gordon Research Conference: Lasers in Medicine and Biology. July 2014. Podium Presentation.
3. AJ Walsh, ME Sanders, RS Cook, MC Skala. "Fluorescence Lifetime Imaging Classifies Breast Cancer Subtypes and Predicts Neoadjuvant Therapy Response in Humans." SPIE Photonics West, San Francisco, California. February 2014. Podium Presentation.
4. AJ Walsh, RS Cook, MC Skala, "Fresh versus frozen tissue preparations for quantitative fluorescence lifetime imaging of metabolic NADH and FAD." SPIE Photonics West, San Francisco, California. February 2014. Podium Presentation.
5. MC Skala. "Metabolic Imaging of Cancer Drug Response." Advanced Imaging Workshop, University of California, Berkeley, CA. January 2014. Podium Presentation.
6. MC Skala. "Optical Metabolic Imaging of Drug Efficacy in Cancer." 7th Annual Workshop on Advanced TCSPC Techniques, Bethesda MD. October 2013. Podium Presentation.

CONCLUSION

These results indicate that OMI in primary breast tumor organoids can provide early, sensitive measures of treatment response. This system can therefore be used to streamline pre-clinical drug development, by reducing the number of animals, cost, and time required to screen new drugs and drug combinations. The feasibility of OMI measurements in organoids derived from primary human breast tumors has also been demonstrated, with the exciting possibility of providing individualized drug screens in patients before the patient is ever treated. This personalized drug planning approach could reduce toxicities of ineffective treatments, and enable improved outcomes through early, effective interventions. The single-cell analysis capabilities of this technique also have the potential to identify drug combinations that will eliminate treatment-resistant sub-populations of cells that are likely to cause metastases.

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1. Walsh, A.J., R.S. Cook, M.E. Sanders, L. Aurisicchio, G. Ciliberto, C.L. Arteaga, and M.C. Skala, *Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer*. Cancer Res, 2014. **accepted**.
2. Walsh, A.J., R.S. Cook, H.C. Manning, D.J. Hicks, A. Lafontant, C.L. Arteaga, and M.C. Skala, *Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer*. Cancer Res, 2013. **73**(20): p. 6164-74.
3. Zhao, Y., E.B. Butler, and M. Tan, *Targeting cellular metabolism to improve cancer therapeutics*. Cell Death Dis, 2013. **4**: p. e532.
4. Narayan, M., J.A. Wilken, L.N. Harris, A.T. Baron, K.D. Kimbler, and N.J. Maihle, *Trastuzumab-induced HER reprogramming in "resistant" breast carcinoma cells*. Cancer research, 2009. **69**(6): p. 2191-4.
5. Walsh, A.J., K.M. Poole, C.L. Duvall, and M.C. Skala, *Ex vivo optical metabolic measurements from cultured tissue reflect in vivo tissue status*. J Biomed Opt, 2012. **17**(11): p. 116015.

APPENDICES

1. AJ Walsh, RS Cook, ME Sanders, L Aurisicchio, G Ciliberto, CL Arteaga, and MC Skala. Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer. Cancer Research (2014), accepted.
2. AJ Walsh, RS Cook, HC Manning, DJ Hicks, A Lafontant, CL Arteaga, and MC Skala. Optical metabolic imaging identifies breast cancer glycolytic levels, sub-types, and early treatment response. Cancer Research (2013)Oct 15;73(20):6164-74.

SUPPORTING DATA

See main text and attached papers.

Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer

Alex J. Walsh¹, Rebecca S. Cook^{2,3,4}, Melinda E. Sanders^{4,5}, Luigi Aurisicchio⁶,
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Running Title: Optical imaging of organoid metabolism predicts drug response

Keywords: fluorescence lifetime imaging, cellular metabolism, NADH, breast cancer, organoid

Notes

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Conflicts of Interest: The authors have no conflicts of interest to disclose.

Word Count: 4998

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Abstract: There is a need for technologies to predict the efficacy of cancer treatment in individual patients. Here we show that optical metabolic imaging of organoids derived from primary tumors can predict therapeutic response of xenografts and measure anti-tumor drug responses in human-tumor derived organoids. Optical metabolic imaging quantifies the fluorescence intensity and lifetime of NADH and FAD, co-enzymes of metabolism. As early as 24 hours after treatment with clinically relevant anti-cancer drugs, the optical metabolic imaging index of responsive organoids decreased ($p < 0.001$) and was further reduced when effective therapies were combined ($p < 5 \times 10^{-6}$), with no change in drug-resistant organoids. Drug response in xenograft-derived organoids was validated with tumor growth measurements *in vivo* and stains for proliferation and apoptosis. Heterogeneous cellular responses to drug treatment were also resolved in organoids. Optical metabolic imaging shows potential as a high-throughput screen to test the efficacy of a panel of drugs to select optimal drug combinations.

Precis: This study demonstrates that cellular-level optical imaging of metabolic co-enzymes resolves early, heterogeneous drug responses within primary tumor organoid cultures that agree with long-term *in vivo* tumor response.

Introduction

With the ever-increasing number of drugs approved to treat cancers, selection of the optimal treatment regimen for an individual patient is challenging. Physicians weigh the potential benefits of the drugs against the side-effects to the patient. Currently, drug regimens for breast cancer are chosen based on tumor expression of several proteins, including estrogen receptor (ER), progesterone receptor (PR), and high levels of human epidermal growth factor receptor 2 (HER2), assessed in the diagnostic biopsy, and drug effectiveness is determined after weeks of treatment from tumor size measurements. A personalized medicine approach would identify the optimal treatment regimen for an individual patient and reduce morbidity from overtreatment.

Current methods to assess therapy response include tumor size, measured by mammography, MRI, or ultrasound. These methods evaluate the regimen that the patient received. Molecular changes induced by anti-tumor drugs precede changes in tumor size and may provide proximal endpoints of drug response. Cellular metabolism may provide biomarkers of early treatment response, because oncogenic drivers typically affect metabolic signaling (1, 2). Indeed FDG-PET has been explored as a predictor of response but lacks the resolution and sensitivity to accurately predict therapy response on a cellular level (3, 4).

Optical metabolic imaging (OMI) provides unique sensitivity to detect metabolic changes that occur with cellular transformation (5-10) and upon treatment with anti-cancer drugs (11). OMI utilizes the intrinsic fluorescence properties of NADH and FAD, co-enzymes of metabolic reactions. OMI endpoints include the optical redox ratio (the fluorescence intensity of NADH divided by the fluorescence intensity of FAD), the NADH and FAD fluorescence lifetimes, and the "OMI index" (a linear combination of these three endpoints). The optical redox ratio

provides a dynamic readout of cellular metabolism (12), with increased redox ratio (NADH/FAD) (8) observed in malignant cells exhibiting the Warburg effect (increased glycolysis despite the presence of oxygen (13)). Fluorescence lifetime values report differences in fluorophore conformation, binding, and microenvironment, such as pH, temperature, and proximity to quenchers such as free oxygen (14). OMI endpoints report early, molecular changes due to anti-cancer drug treatment (11) and are powerful biomarkers of drug response.

Primary tumors can be cultured *ex vivo* as organoids, which contain the malignant tumor cells and the supporting cells from the tumor environment, such as fibroblasts, leukocytes, endothelial cells, and hematopoietic cells (15). Interactions between cancer cells and stromal cells have been shown to mediate therapeutic resistance in tumors (16). Therefore, organoid cultures provide an attractive platform to test cancer cell response to drugs in a relevant, "body-like" environment. Furthermore, multiple organoids can be generated from one biopsy, enabling high-throughput tests of multiple drug combinations with a small amount of tissue.

OMI of primary tumor organoids enables high-throughput screening of potential drugs and drug combinations to identify the most effective treatment for an individual patient. Here, we validate OMI in primary tumor organoid cultures as an accurate, early predictor of *in vivo* tumor drug response in mouse xenografts, and present the feasibility of this approach on primary human tissues. The cellular-resolution of this technique also allows for subpopulations of cells to be tracked over time with treatment, to identify therapies that affect all cells in a heterogeneous population.

Materials and Methods

Mouse xenografts. This study was approved by the Vanderbilt University Animal Care and Use Committee and meets the National Institutes of Health guidelines for animal welfare. BT474

cells or HR6 cells (10^8) in 100 μ l Matrigel were injected in the inguinal mammary fat pads of female athymic nude mice (J:NU; Jackson Laboratories). Tumors grew to $\geq 200\text{mm}^3$. Tumor-bearing mice were treated twice weekly with the following drugs: control human IgG (10 mg/kg, IP; R&D Systems), trastuzumab (10 mg/kg, IP; Vanderbilt Pharmacy), paclitaxel (2.5 mg/kg, IP; Vanderbilt Pharmacy), XL147 (10 mg/kg, oral gavage; Selleckchem), trastuzumab + XL147, trastuzumab + paclitaxel, trastuzumab + paclitaxel + XL147. Tumor volume was calculated from caliper measurements of tumor length (L) and width (W), $(L \cdot W^2)/2$ twice a week.

Primary human tissue collection. This study was approved by the Vanderbilt University Institutional Review Board and informed consent was obtained from all subjects. A primary tumor biopsy, removed from the tumor mass after surgical resection, was provided by an expert breast pathologist (M.E.S.). The tumor was placed immediately in sterile DMEM, transported on ice to the laboratory (~5 minute walk), and generated into organoids within 3 hours of tissue resection. Pathology and receptor status of the tissue were obtained from the patient's medical chart.

Organoid generation and culture. Breast tumors (xenografts and primary) were washed three times with PBS. Tumors were mechanically dissociated into 100-300 μ m macro-suspensions in 0.5 ml PMEC media (DMEM:F12 + EGF (10 ng/ml) + hydrocortisone (5 μ g/ml) + insulin (5 μ g/ml) + 1% penicillin: streptomycin) by cutting the tissues with a scalpel or by spinning in a C-tube (Miltenyi Biotec). Macro-suspension solutions were combined with Matrigel in a 1:2 ratio, and 100 μ l of the solution was placed on cover slips. The gels solidified at room temperature for 30 minutes and then for 1 hour in the incubator. The gels were over-lain with PMEC media supplemented with drugs. The following *in vitro* drug dosages were used to replicate *in vivo*

doses (17-19): control (control human IgG + DMSO), trastuzumab (25 $\mu\text{g/ml}$), paclitaxel (0.5 μM), XL147 (25 nM), tamoxifen (2 μM), fulvestrant (1 μM), and A4 (10 $\mu\text{g/ml}$, Takis, Inc.).

Fluorescence lifetime instrumentation. Fluorescence lifetime imaging was performed on a custom built multi-photon microscope (Prairie Technologies), as described previously (11, 20). Excitation and emission light were coupled through a 40X oil immersion objective (1.3 NA) within an inverted microscope (Nikon, TiE). A titanium:sapphire laser (Coherent Inc.) was tuned to 750 nm for NADH excitation (average power 7.5-7.9 mW) and 890 nm for FAD excitation (average power 8.4-6 mW). Bandpass filters, 440/80 nm for NADH and 550/100 nm for FAD, isolated emission light. A pixel dwell time of 4.8 μs was used to acquire 256x256 pixel images. Each fluorescence lifetime image was collected using time correlated single photon counting electronics (SPC-150, Becker and Hickl) and a GaAsP PMT (H7422P-40, Hamamatsu). Photon count rates were maintained above 5×10^5 for the entire 60s image acquisition time, ensuring no photobleaching occurred. The instrument response full width at half maximum was 260 ps as measured from the second harmonic generation of a urea crystal. Daily fluorescence lifetime validation was confirmed by imaging of a fluorescent bead (Polysciences Inc). The measured lifetime of the bead (2.1 ± 0.06 ns) concurs with published values (10, 20, 21).

Organoid imaging. Fluorescence lifetime images of organoids were acquired at 24, 48, and 72 hours post-drug treatment. Organoids were grown in 35-mm glass-bottom petri dishes (MatTek Corp) and imaged directly through the coverslip on the bottom of the petri dish. Six representative organoids from each treatment group were imaged. The 6 organoids imaged contained collectively approximately 60-300 cells per treatment group for statistical and subpopulation analyses. First, an NADH image was acquired and a subsequent FAD image was acquired of the exact same field of view.

Immunofluorescence. A previously reported protocol (22) was adapted for immunofluorescent staining of organoids. Briefly, gels were washed with PBS and fixed with 2 ml 4% paraformaldehyde in PBS. Gels were washed with PBS, and then 0.15M glycine in PBS was added for 10 minutes. Gels were washed in PBS, and then added to 0.02% Triton X-100 in PBS. Gels were washed with PBS then overlain with 1% fatty acid-free BSA, 1% donkey serum in PBS. The next day, the solution was removed and 100 μ l of antibody solution (diluted antibody in PBS with 1% donkey serum) was added to each gel. The gels were incubated for 30 minutes at room temperature, washed in PBS 3 times, and then incubated in 100 μ l of secondary antibody solution for 30 minutes at room temperature. The gels were washed in PBS 3 times, washed in water 2 times, and then mounted to slides using 30 μ l of ProLong Antifade Solution (Molecular Probes).

The primary antibodies used were anti-cleaved caspase 3 (Life Technologies) and anti-Ki67 (Life Technologies). Both were diluted at 1:100. A goat anti-rabbit IgG FITC secondary antibody was used (Life Technologies). FITC fluorescence was obtained by excitation at 980 nm on the multiphoton microscope described above, and a minimum of 6 organoids were imaged. Positive staining of cleaved caspase 3 and Ki67 was confirmed by staining mouse thymus and mouse small intestine, respectively. Immunofluorescence images were quantified by manual counting of the total number of cells and the number of positively stained cells in each field of view. Immunofluorescence results were presented as percentage of positively stained cells, quantified from six organoids, approximately 200 cells.

Generation of OMI endpoint images: Photon counts for 9 surrounding pixels were binned (SPCImage). Fluorescence lifetime components were extracted from the photon decay curves by deconvolving the measured system response and fitting the decay to a two component model,

$I(t) = \alpha_1 \exp^{-t/\tau_1} + \alpha_2 \exp^{-t/\tau_2} + C$, where $I(t)$ is the fluorescence intensity at time t after the laser pulse, α_1 and α_2 are the fractional contributions of the short and long lifetime components, (i.e. $\alpha_1 + \alpha_2 = 1$), τ_1 and τ_2 are the fluorescence lifetimes of the short and long lifetime components, and C accounts for background light. A two-component decay was used to represent the lifetimes of the free and bound configurations of NADH and FAD (10, 23, 24) and yielded the lowest Chi² values (0.99-1.1), indicative of optimal fit. Matrices of the lifetime components were exported as ascii files for further processing in Matlab.

Automated image analysis software. To streamline cellular-level processing of organoid images, an automated image analysis routine, as previously described (25), was used in Cell Profiler in Matlab. Briefly, a customized threshold code identified pixels belonging to nuclear regions that were brighter than background but not as bright as cell cytoplasms. These nuclear pixels were smoothed and the resulting round objects between 6 and 25 pixels in diameter were segmented and saved as the nuclei within the image. Cells were identified by propagating out from the nuclei. An Otsu Global threshold was used to improve propagation and prevent propagation into background pixels. Cell cytoplasms were defined as the cells minus the nuclei. Cytoplasm values were measured from each OMI image (redox ratio, NADH τ_m , NADH τ_1 , NADH τ_2 , NADH α_1 , FAD τ_m , FAD τ_1 , FAD τ_2 , FAD α_1).

Computation of OMI index. The redox ratio, NADH τ_m , and FAD τ_m were norm-centered across cell values from all treatment groups within a sample, resulting in unit-less parameters with a mean of 1. The OMI index is the linear combination of the norm-centered redox ratio, NADH τ_m , and FAD τ_m with the coefficients (1, 1, -1), respectively, computed for each cell. The three endpoints, redox ratio, NADH τ_m , and FAD τ_m are independent variables (11) and are

thus weighted equally. The signs of the coefficients were chosen to maximize difference between control and drug-responding cells.

Subpopulation analysis. Subpopulation analysis was performed by generating histograms of all cell values within a group as previously reported (11). Each histogram was fit to a 1, 2, and 3 component Gaussian curves. The lowest Akaike information criterion (AIC) signified the best fitting probability density function for the histogram (26). Probability density functions were normalized to have an area under the curve equal to 1.

Statistical tests. Differences in OMI endpoints between treatment groups were tested using a student's t-test with a Bonferroni correction. An α significance level less than 0.05 was used for all statistical tests.

Results

Response of BT474 organoids to a panel of anti-cancer drugs

Validation of an organoid-OMI screen for drug response was first tested in two isogenic HER2-amplified breast cancer xenografts. BT474 xenografts are sensitive to the HER2 antibody trastuzumab, while HR6 xenografts, derived as a sub-line of BT474, are trastuzumab-resistant. The following single drugs and drug combinations were tested: paclitaxel (P, chemotherapy), trastuzumab (H, anti-HER2 antibody), XL147 [X, phosphatidylinositol-3 kinase (PI3K) small molecule inhibitor] (27), H+P, H+X, and H+P+X. Paclitaxel and trastuzumab are standard-of-care drugs, and XL147 is in clinical trials and preclinical studies support combination therapy of XL147 with trastuzumab for patients who have developed a resistance to trastuzumab (27, 28).

Representative redox ratio, NADH τ_m , and FAD τ_m images of BT474 xenograft-derived organoids demonstrate mixed multicellular morphology and highlight the sub-cellular resolution

of this technique (Fig. 1A-F). A longitudinal study of tumor growth demonstrated that the BT474 xenografts responded to each treatment arm (Fig. 1G), with significant reduction in tumor volume, as determined from caliper measurements, on day 7 for all treatment groups except trastuzumab, which had significant reduction on day 11 (Fig. 1H).

A composite endpoint, the OMI index, was computed as a linear combination of the mean-normalized optical redox ratio, NADH τ_m , and FAD τ_m for each cell. After 24 hr of treatment, the OMI index was significantly reduced in all treated BT474 organoids, compared to the control ($p < 0.05$, Fig. 1I). By 72 hr, the OMI index decreased further in all treatment groups ($p < 5 \times 10^{-7}$, Fig. 1J). The redox ratio, NADH τ_m , and FAD τ_m values showed similar trends (Supplementary Fig. 1). Changes in short and long lifetime values and in the portion of free NADH or FAD contributed to the changes in τ_m (Supplementary Table 1).

The high resolution capabilities of OMI allowed single cell analysis and population modeling for quantification of cellular subpopulations with varying OMI indices. Visual inspection of cell morphology suggested that the majority of cells are tumor epithelial cells; stromal cells with obvious morphological differences were eliminated from the analysis. Population density modeling of cellular distributions of the OMI index revealed two populations with high and low OMI index values in all of the BT474 treated organoids at 24 hr (Fig. 1K, Supplementary Fig. 2). By 72 hr, the XL147, H+P, H+X, and H+P+X treated organoids have a single population with narrower peaks (Fig. 1L, Supplementary Fig. 2). The trastuzumab treated organoids have two populations at 72 hr, both lower than the mean OMI index of the control organoids (Fig. 1L). Immunofluorescent staining of cleaved caspase-3 and Ki67 of BT474 organoids treated for 72 hr confirmed increased apoptosis and decreased proliferation in drug

treated organoids, with the greatest increases in cell death with combined treatments (Fig. 1M-N).

Response of HR6 organoids to a panel of anti-cancer drugs

Next, the OMI-organoid screen was tested on trastuzumab-resistant HR6 xenografts (29). Representative images show HR6 organoid morphology and spatial distributions of OMI endpoints (Fig. 2A-F). These HER2 overexpressing tumors had continued growth with trastuzumab treatment (Fig. 2G). Treatment with paclitaxel and XL147 initially caused HR6 tumor regression ($p < 0.05$ on day 10 for XL147 and on day 14 for paclitaxel) but then resumed growth (Fig. 2G-H). Mice treated with the H+P, H+X, and H+P+X combination therapies exhibited sustained HR6 tumor reduction (Fig. 2G-H).

After 24 hr of treatment, significant reductions in the OMI index were detected in HR6 organoids treated with paclitaxel, XL147, H+P, H+X, and H+P+X ($p < 0.05$, Fig. 2I). At 72 hr, the OMI index of the paclitaxel and XL147 treated organoids was significantly greater than that of the control organoids ($p < 0.05$, Fig. 2J), consistent with the recovery of HR6 tumor growth after prolonged therapy (Fig. 2G). The organoids treated with drug combinations (H+P, H+X, H+P+X) continued to have significantly lower OMI index values ($p < 10^{-6}$) at 72 hr, compared to untreated controls. Individual OMI endpoints showed similar trends (Supplementary Fig. 3, Supplementary Table 2). Subpopulation analysis revealed two subpopulations in the OMI index for all treated groups except for trastuzumab at 24 hr (Fig. 2K, Supplementary Fig. 4). By 72 hr, the paclitaxel and XL147 treated organoids had a single population (Fig. 2L, Supplementary Fig. 4). Immunofluorescent staining of cleaved caspase 3 of organoids treated for 72 hr revealed increased cell death in HR6 organoids treated with H+P, H+X and H+P+X ($p < 0.05$, Fig. 2M).

The percentage of Ki67 positive cells at 72 hr decreased with paclitaxel, H+P, H+X, and H+P+X treatment ($p < 0.005$, Fig. 2N).

OMI endpoints identify breast cancer subtypes

We tested these methods on primary breast cancer biopsies obtained from surgical resection. Tumors were obtained fresh from de-identified mastectomy specimens not required for further diagnostic purposes, and dissociated into organoids within 1-3 hr post-resection. Cancer drugs were added and organoids were imaged with OMI. Representative redox ratio, NADH τ_m , and FAD τ_m images (Fig. 3) demonstrate the varying morphology of organoids derived from ER+, HER2+ and triple negative breast cancers.

When quantified, the OMI endpoints differed between cancer subtypes. In immortalized cell lines, the redox ratio was elevated in ER+/HER2- cells and was greatest in HER2+/ER- cells ($p < 5 \times 10^{-5}$, Fig. 4A). Similarly, NADH τ_m was increased in immortalized ER+/HER2- and HER2+/ER- breast cancer cells as compared to triple negative breast cancer (TNBC) cells ($p < 5 \times 10^{-8}$, Fig. 4B). FAD τ_m was greatest in ER+/HER2- cells ($p < 0.05$, Fig. 4C). Overall, the OMI index was lowest in TNBC and greatest in HER2+/ER- cells ($p < 5 \times 10^{-8}$, Fig. 4D), suggesting that HER2 and ER expression influence cellular metabolism.

Similar trends were observed for the OMI endpoints in organoids derived from primary breast tumor specimens cultured under basal conditions. The redox ratio was increased in organoids from ER+/HER2- tumors and was greatest in HER2+/ER- organoids ($p < 5 \times 10^{-12}$, Fig. 4E, Supplementary Table 3). Likewise, NADH τ_m increased with ER and HER2 expression ($p < 5 \times 10^{-8}$, Fig. 4F). FAD τ_m was increased in ER+ organoids and reduced in HER2+ organoids

($p < 0.05$, Fig. 4G). The OMI index was lowest for TNBC, and greatest for HER2+ organoids ($p < 5 \times 10^{-3}$, Fig. 4H).

Organoid response of ER+ primary human tumors

Organoids were generated from four ER positive (HER2-negative) primary human tumors and treated with the chemotherapeutic drug paclitaxel, the selective ER modulator tamoxifen, the HER2 antibody trastuzumab and the pan-PI3K inhibitor XL147. Organoids derived from the first ER+ tumor had significantly reduced OMI index values upon treatment with paclitaxel, tamoxifen, XL147, H+X, H+P+T, H+P+X and H+P+T+X for 72 hr ($p < 5 \times 10^{-5}$, Fig. 5A). Immunofluorescence of cleaved caspase-3 showed increased cell death in parallel organoids treated for 72 hr with paclitaxel, tamoxifen, XL147, H+X, H+P+T, H+P+X, and H+P+T+X (Fig. 5B). Subpopulation analysis revealed less variability (narrower histogram peaks) within responsive treatment groups compared to the cells of control and trastuzumab-treated organoids (Fig. 5C, Supplementary Fig. 5). Corresponding OMI endpoints showed similar trends (Supplementary Fig. 6, Supplementary Table 4).

Organoids derived from a second ER+ tumor responded similarly. The OMI index decreased upon treatment with paclitaxel, tamoxifen, H+P, P+T and H+P+T at 72 hr ($p < 5 \times 10^{-5}$, Fig. 5D). Subpopulation analysis revealed a single population of control cells that shifted to lower OMI indexes with paclitaxel, tamoxifen, H+P, P+T, and H+P+T treatments (Fig. 5E, Supplementary Fig. 7). Corresponding OMI endpoints showed similar trends (Supplementary Fig. 8, Supplementary Table 5).

The third and fourth ER+ clinical samples yielded organoids with variable responses to treatment. Organoids derived from the third patient had significant reductions in OMI index after

24 hr treatment with tamoxifen, XL147, H+X, and H+P+T+X treatments ($p<0.005$, Fig. 5F). Subpopulation analysis revealed two populations with high and low OMI index values for the H+P and paclitaxel-treated organoids (Fig. 5G; Supplementary Fig. 9). Two populations, both with mean OMI index values less than that of the control organoids, were apparent in the organoids treated with XL147 and with H+P+T+X (Fig. 5G, Supplementary Fig. 9). Organoids from the fourth ER+ patient had reduced OMI indices following treatment with XL147, H+P, H+X, H+P+X, H+P+T and H+P+T+X for 72 hr ($p<0.01$, Fig. 5H). Subpopulation analysis of cells from these organoids revealed single populations with shifted mean OMI indices for all treatments except tamoxifen, H+P, and H+X, which had two populations (Fig. 5I, Supplementary Fig. 10). Corresponding OMI endpoints showed similar trends (Supplementary Fig. 11-12, Supplementary Tables 6-7).

Organoid Response of HER+ and TNBC primary human tumors

OMI was also performed on organoids derived from HER2+ (ER negative) and TNBC specimens. Organoids derived from the HER2+ primary tumor were treated with the ER down-regulator fulvestrant, the HER2 antibody trastuzumab, and the anti-ErbB3 antibody A4 (30). The OMI index was significantly decreased in the organoids treated for 24 hr with trastuzumab and A4 ($p<0.005$, Fig. 6A). Subpopulation analysis revealed shifts in the mean OMI index values with these treatments within a single population of cells (Fig. 6B). Organoids derived from the TNBC specimen were treated with tamoxifen, the HER2 antibody trastuzumab, and the combination of trastuzumab plus tamoxifen (H+T). No significant changes were observed with these treatments in TNBC organoids after 24 hr ($p>0.3$, Fig. 6C). Subpopulation analysis revealed a single population of cells from TNBC organoids (Fig. 6D). Corresponding OMI endpoints showed similar trends (Supplementary Fig. 7-8, Supplementary Tables 8-9).

Discussion

Primary tumor organoids are an attractive platform for drug screening because they are grown from intact biopsies, thus maintaining the tumor cells within the same tumor microenvironment (15). OMI is sensitive to early metabolic changes, achieves high resolution to allow analysis of tumor cell heterogeneity, and uses endogenous contrast in living cells for repeated measurements and longitudinal studies (11). The OMI index is a holistic reporter of cellular metabolism because the redox ratio and NADH and FAD lifetimes are independent measurements (11). The mean lifetime captures not only changes in free-to-bound protein ratios but also preferred protein binding and relative concentrations of NADH to NADPH (31). Cancer drugs have been shown to down-regulate certain metabolism enzymes; for example, trastuzumab down-regulates lactate dehydrogenase in breast cancer and paclitaxel resistant cells have been shown to have more lactate dehydrogenase expression and activity (32). The OMI index captures these drug-induced changes in metabolism enzyme activity. Organoids remain viable with stable OMI endpoints in controlled culture conditions (33), thus making them an attractive system to evaluate tumor response to drugs. We used OMI to assess the response of primary breast tumor organoids to a panel of clinically relevant anti-cancer agents used singly or in combination. Early OMI-measured response in organoids (24-72 hr post-treatment) corroborated with standard tumor growth curves in xenografts, and the feasibility of this approach was confirmed in organoids derived from primary human breast tumors.

The OMI index was first evaluated as a reporter of tumor response in organoids derived from BT474 (ER+/HER2+) xenografts. Significant reductions in OMI index upon treatment with paclitaxel, trastuzumab, XL147 and combinations thereof, at both 24 and 72 hr, correlated with reduction of tumor growth (Fig. 1). Biochemically, cellular rates of glycolysis, and NADH and

FAD protein-binding decrease with drug treatment in responsive cells (32), resulting in decreased redox ratios and NADH τ_m , and increased FAD τ_m in agreement with the decreased OMI index observed in drug-treated BT474 organoids. Significant reductions in tumor growth occurred 7-11 days post-treatment initiation whereas the OMI index detected response at 24-72 hr post-treatment. Cellular analysis revealed an initial heterogeneous response among cells within organoids treated with paclitaxel and H+P at 24 hr, which, by 72 hr, became a uniform response. The heterogeneity of trastuzumab treated BT474 organoids persisted over 72 hr, suggesting an intrinsic subpopulation more susceptible to acquire drug resistance. This heterogeneity was not seen in the combination treatments, suggesting the combination treatments trump this drug resistance-prone subpopulation. OMI measured response corroborated with increased cell death and decreased proliferation due to single and combination drug treated organoids, measured with destructive post-mortem techniques. The XL147-treated BT474 organoids have a much lower OMI index at 72 hr, but only a modest increase in cleaved caspase 3 activity. The same decrease was not observed in the HR6 cells that have alternative metabolism pathways activated due to their acquired resistance to trastuzumab. The OMI index detects changes in cellular metabolism that predict drug efficacy, but do not necessarily correlate with IHC.

In the current standard of care, patients with innate drug resistance are not identified *a priori*. We tested the capabilities of OMI to predict drug resistance using trastuzumab-resistant HR6 (ER+/HER2+) tumors (29). XL147 is a novel PI3K inhibitor under investigation for combined therapy with trastuzumab to improve response of resistant tumors (27). Significant reductions in the OMI index of HR6 organoids treated for 72 hr identified drug combinations (H+X, H+P, and H+P+X) that induced a sustained reduction in tumor growth *in vivo* (Fig. 2).

The reduction in tumor growth upon treatment with H+X was consistent with previous reports of greater anti-tumor effects of the combination over trastuzumab and XL147 alone (27). Subpopulation analysis revealed multiple responses within the HR6 organoids after treatment with single drugs and combinations, suggesting increased heterogeneity compared to the parental BT474 organoids.

The OMI index of paclitaxel and XL147 treated HR6-organoids initially decreased at 24 hr, and then increased at 72 hr, mirroring the tumor growth in mice after prolonged therapy, and indicating that the adaptations that allow HR6 cells to survive trastuzumab treatment also affect response to additional drugs. This relapse of HR6 tumors treated with paclitaxel and XL147 was not apparent until 2-3 weeks of drug treatment; yet, the OMI index identified a resistant population within both paclitaxel and XL147 treated organoids at 24 hours and showed a selection of this population by 72hr. Subpopulation analysis of the paclitaxel and XL147 treated HR6 organoids revealed heterogeneous responses at 24 hr, suggesting that OMI is capable of early detection of resistant cells within a heterogeneous tumor. These results indicate that OMI of primary tumor organoids is able to identify heterogeneous responses within tumors on a cellular level, and potentially guide therapy selection early for maximal response. The ability to detect innate resistance at a cellular level prior to treatment may provide leads for identification of drugs that target such refractory subpopulations before they are selected by the primary therapy.

We next examined the feasibility of this approach utilizing fresh tumor biopsies obtained from primary tumor surgical resections. OMI measurements *in vivo* and corresponding measurements from freshly excised tissues within 8 hr of surgery are statistically identical (20), providing ample time for specimen acquisition and transport to the laboratory. The morphology

of organoids differed among patients and within breast cancer subtypes (Fig. 3), demonstrating a greater heterogeneity within primary tumors compared to xenografts.

Previously published studies report differences in OMI endpoints due to the presence or absence of ER and HER2 (8, 11, 34). Both ER and HER2 signaling pathways can influence metabolism: ER by inducing increased glucose transport (1), and HER2 through activation of PI3K (2), among other signal transducers. We compared OMI endpoints from immortalized cells and human tissue-derived organoids of three subtypes of breast cancer: ER+, HER2-overexpressing, and triple negative breast cancer (TNBC). The OMI index of immortalized cell lines increased with ER expression and was highest in HER2 overexpressing cells (consistent with prior studies (11)), and these trends were replicated in organoids derived from primary human tumors. Notably, NADH τ_m was significantly increased ($p < 0.05$) in the HER2+ organoids compared to ER+ organoids, but this trend was not observed in the immortalized cell lines. This difference could be due to molecular changes induced by the immortalization process, media components, primary tumor heterogeneity, and/or the heterogeneity within a primary breast tumor. Regardless, the results shown (Fig. 4) suggest breast cancer subtypes, ER+, HER2+ and TNBC, have different OMI profiles.

Organoids derived from human breast tumors were treated with a panel of breast cancer drugs (Fig. 5-6). Differences in the drug response of these organoids suggest heterogeneity across ER+/HER- tumors. Organoids from one of the four ER+ tumors did not exhibit reduced OMI indices after treatment with tamoxifen. Organoids derived from two of the four ER+ samples did not have reduced OMI indices after paclitaxel treatment. These variable responses are consistent with variable responses seen with these drugs in the clinic (35-38). None of the organoids had reduced OMI indices with trastuzumab, which is expected because the organoids

were derived from HER2 negative tumors. Generally, the OMI index was reduced further upon treatment with drug combinations, supporting the use of drug combinations clinically.

Subpopulation analysis revealed cells within organoid treatment groups that exhibit different OMI indices after treatment, suggesting that subpopulations of cells with different drug sensitivities preexist and develop within primary tumors. Some of these cells may represent the cancer stem-like population with increased renewal capacity, metastatic potential, and drug resistance (39). The populations of organoids derived from human tumors have more variability (broader population curves) than those derived from xenografts, reflecting an inherent greater heterogeneity within primary tumors. This corroborates previous reports (40) of greater intratumoral heterogeneity in primary tumors than in xenografts derived from clonal cell lines. Thus, OMI imaging allows identification of heterogeneous cellular response to drug treatment in a dynamic population, which potentially enables drug selection to maximize therapeutic efficacy.

Organoids derived from HER2+/ER- and TNBC primary tumors have OMI responses consistent with their clinical characteristics: reduced OMI index with trastuzumab treatment and no change with fulvestrant (ER antagonist) treatment in the HER2+/ER- organoids (41), and no OMI index reductions after treatment with trastuzumab or tamoxifen in the TNBC organoids (42, 43) (Fig. 6a,c). HER3 is an emerging target for breast cancer (30, 44) and the anti-HER3 antibody A4 reduced the OMI index of HER2+/ER- organoids.

The results of this study support the validity of OMI for monitoring organoid response to anti-cancer drugs. We demonstrate high selectivity of the OMI index to directly measure drug response of organoids derived from breast cancer xenografts to single anti-cancer drugs and their combinations, and validated OMI measured response with gold standard tumor growth in two

xenograft models. We have shown that the OMI index measured in primary tumor organoids resolves response and non-response within 72 hours, compared to the 3 weeks required to resolve this response with tumor size measurements. Further, we extend this approach and generate drug response information from organoids derived from three subtypes of primary human tumors, TNBC, ER+, and HER2+. The high resolution of OMI allows subpopulation analysis for identification of heterogeneous tumor response to drugs in dynamic tumor cell populations. Altogether, these results suggest that OMI of primary tumor organoids may be a powerful test to predict the action of anti-cancer drugs and tailor treatment decisions accordingly.

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Figure Captions

Figure 1. OMI of organoids derived from trastuzumab-responsive xenografts. A. Redox ratio image of a control BT474 (ER+/HER2+) organoid at 72hr. Scale bar is 100 μ m. B. NADH τ_m image of a control BT474 organoid at 72hr. C. FAD τ_m image of a control BT474 organoid at 72hr. D. Redox ratio image of a trastuzumab (anti-HER2) plus paclitaxel (chemotherapy) plus XL147 (anti-PI3K) (H+P+X) treated BT474 organoid at 72hr. E. NADH τ_m image of a trastuzumab plus paclitaxel plus XL147 (H+P+X) treated BT474 organoid at 72hr. F. FAD τ_m image of a trastuzumab plus paclitaxel plus XL147 (H+P+X) treated BT474 organoid at 72hr. G. Tumor growth response of BT474 tumors grown in athymic nude mice and treated with single and combination treatments. H. Table of earliest detectable ($p < 0.05$) reduction in tumor size for control vs. treated mice. I. OMI index decreases in BT474 organoids treated with single and combination therapies at 24 hr. J. OMI index of BT474 organoids treated for 72hr. Red bars denote $p < 0.05$ for treated organoids vs. control. K. Population density modeling of the mean OMI index per cell in control, paclitaxel, trastuzumab, and H+P+X treated organoids at 24 hr. L. Population density modeling of the OMI index for control, paclitaxel, trastuzumab, and H+P+X BT474 organoids treated for 72hr. M. Immunofluorescence staining of cleaved caspase 3 in control and treated BT474 organoids at 72hr. N. Immunofluorescence staining of Ki67 in control and treated BT474 organoids at 72hr. * $p < 0.05$.

Figure 2. OMI of organoids derived from trastuzumab-resistant xenografts. A. Redox ratio image of a control HR6 (ER+/HER2+) organoid at 72hr. Scale bar is 100 μ m. B. NADH τ_m image of a control HR6 organoid at 72hr. C. FAD τ_m image of a control HR6 organoid at 72hr. D. Redox ratio image of a trastuzumab (anti-HER2) plus paclitaxel (chemotherapy) plus XL147

(anti-PI3K) (H+P+X) treated HR6 organoid at 72hr. E. NADH τ_m image of an H+P+X treated HR6 organoid at 72hr. F. FAD τ_m image of an H+P+X treated HR6 organoid at 72hr. G. Tumor growth response of HR6 tumors grown in athymic nude mice and treated with single and combination treatments. H. Table of earliest detectable ($p < 0.05$) reduction in tumor size for control vs. treated mice. * Denotes tumors that initially shrank and then grew. NS, not significant. I. OMI index initially decreases in HR6 organoids treated with paclitaxel, XL147, and combination therapies at 24 hr. J. OMI index of HR6 organoids treated for 72hr. Red bars indicate significant reductions in OMI index, $p < 0.05$, for treated organoids vs. control. Blue bars indicate significant increases in OMI index, $p < 0.05$, for treated organoids vs. control. K. Population density modeling of the mean OMI index per cell in control, paclitaxel, trastuzumab, and H+P+X organoids at 24 hr. L. Population density modeling of the OMI index for HR6 control, paclitaxel, trastuzumab, and H+P+X organoids treated for 72 hr. M. Immunofluorescence staining of cleaved caspase 3 in control and treated HR6 organoids at 72hr. N. Immunofluorescence staining of Ki67 in control and treated HR6 organoids at 72hr. * $p < 0.05$

Figure 3. Representative redox ratio, NADH τ_m , and FAD τ_m images of organoids derived from primary, human breast tumors. Redox ratio (NADH/FAD; first row), NADH τ_m (second row), and FAD τ_m (third row) images of organoids generated from primary human breast tissue obtained from resection surgeries. TBNC = Triple negative breast cancer. Scale bar is 100 μm .

Figure 4. OMI endpoints differ among breast cancer subtypes. A. Redox ratio (NADH/FAD) of TNBC cells (MDA-MB-231), ER+ (HER2-negative) cells (MCF7), and HER2+ (ER-negative) cells (SKBr3, BT474, MDA-MB-361). B. NADH τ_m of TNBC, ER+,

and HER2+ immortalized cell lines. C. FAD τ_m of TNBC, ER+, and HER2+ immortalized cell lines. D. OMI index increases in ER+ and HER2+ immortalized cell lines. E. Redox ratio (NADH/FAD) of organoids derived from triple negative, ER+ (HER2-negative), and HER2+ (ER-negative) primary human tumors. F. NADH τ_m of organoids derived from triple negative, ER+, and HER2+ primary human tumors. G. FAD τ_m of organoids derived from triple negative, ER+, and HER2+ primary human tumors. H. OMI index of organoids derived from triple negative, ER+, and HER2+ primary human tumors. * $p < 0.05$

Figure 5. OMI index measures drug response and heterogeneous populations in ER+ primary tumor derived organoids. A. OMI index of organoids derived from an ER+ (HER2-negative) primary human tumor decreases with paclitaxel (chemotherapy), tamoxifen (ER antagonist), XL147 (anti-PI3K) and combined therapies at 72hr. Light gray bars indicate significant ($p < 0.05$) reductions in OMI index with treatment, versus control organoids. B. Quantification of immunofluorescence staining of cleaved caspase 3 for organoids derived from the same tumor sample as in (A) and treated for 72hr. C. Population density modeling of the control, H+X, and H+P+T+X treated organoids presented in (A). D. OMI index is reduced with paclitaxel, tamoxifen, and combined treatments in organoids derived from a different ER+ patient at 72hr. E. Population density modeling of the control, tamoxifen, and H+P+T treated organoids presented in (D). F. OMI index is reduced in organoids from a third ER+ patient treated with tamoxifen, XL147, H+X, and H+P+T+X at 24hr. G. Population density modeling of the control, XL147, and H+P+T+X treated organoids presented in F. H. Organoids derived from a fourth ER+ patient have significant reductions in OMI index when treated with XL147 and combination therapies at 72hr. I. Population density modeling of the control, tamoxifen,

and H+P+X treated organoids in (H) reveals multiple populations with tamoxifen treatment. *
p<0.05.

Figure 6. OMI index detects response of HER2+ organoids to trastuzumab and resolves no response in TNBC. A. Organoids derived from a HER2+(ER-negative) clinical tumor have reduced OMI indices with trastuzumab (anti-HER2) and A4 (anti-ErbB3) treatment, and no change with fulvestrant treatment (ER antagonist) at 24hr. Light gray bars signify significant reductions in OMI index due to drug treatment compared to control organoids (*p<0.05). (B). Population density modeling of the organoids derived from a HER2+ tumor reveals single populations. C. Organoids derived from a TNBC tumor have no significant changes (p>0.3) in OMI index with treatment of targeted therapies, tamoxifen (ER antagonist) and trastuzumab at 48hr. D. Population density modeling reveals single populations within the TNBC organoids.

Figure-1 (Skala)

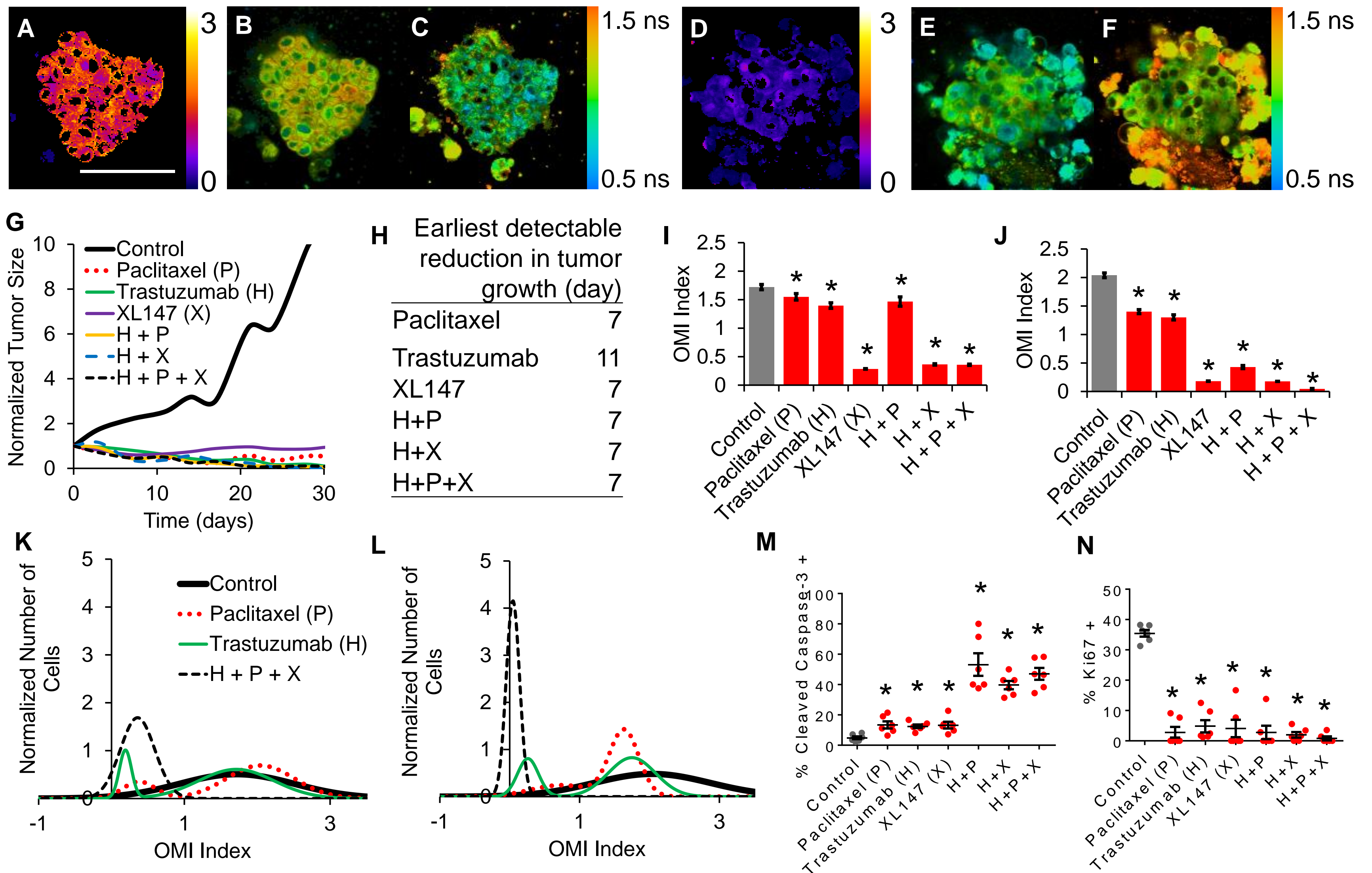


Figure-2 (Skala)

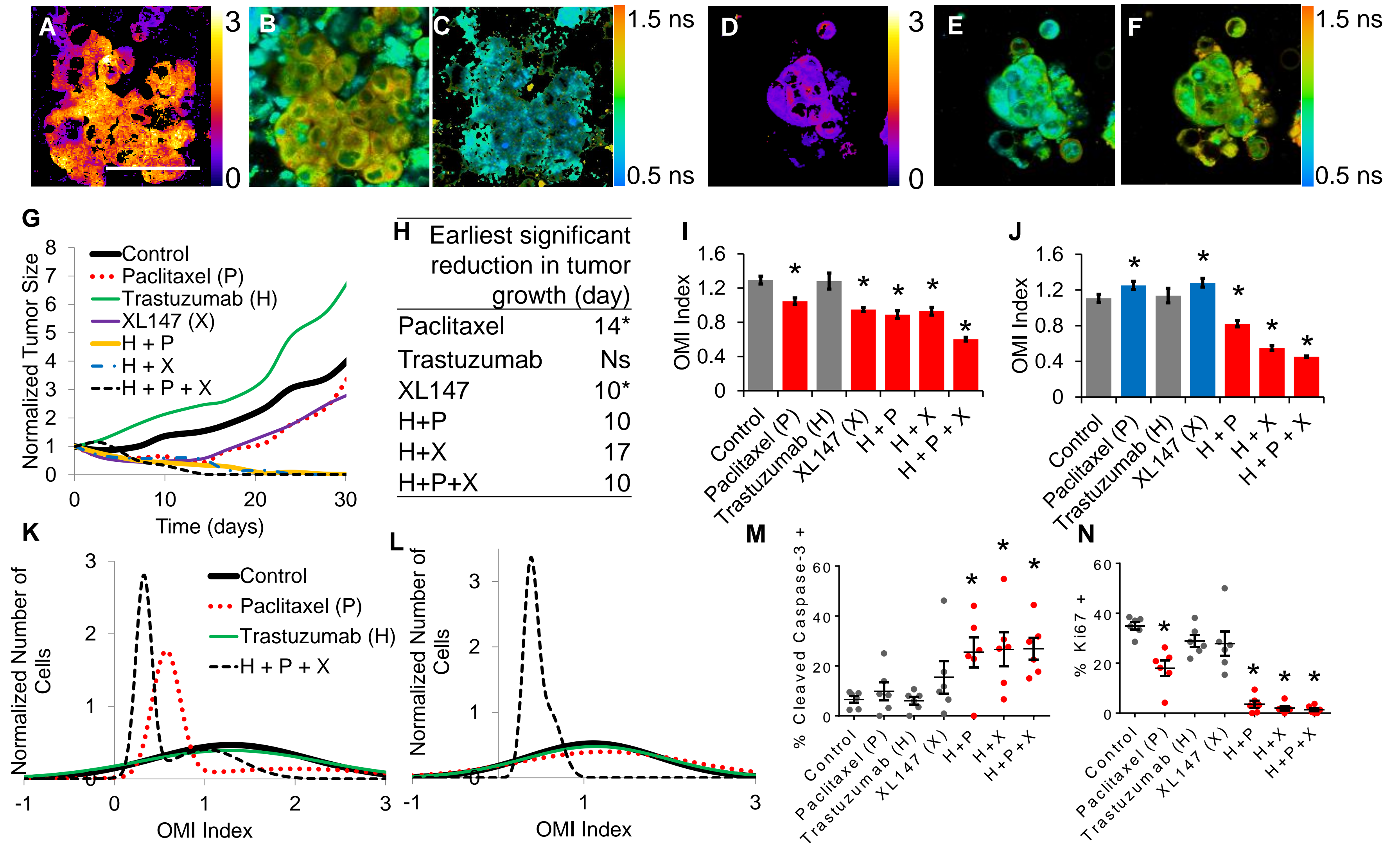


Figure-3 (Skala)

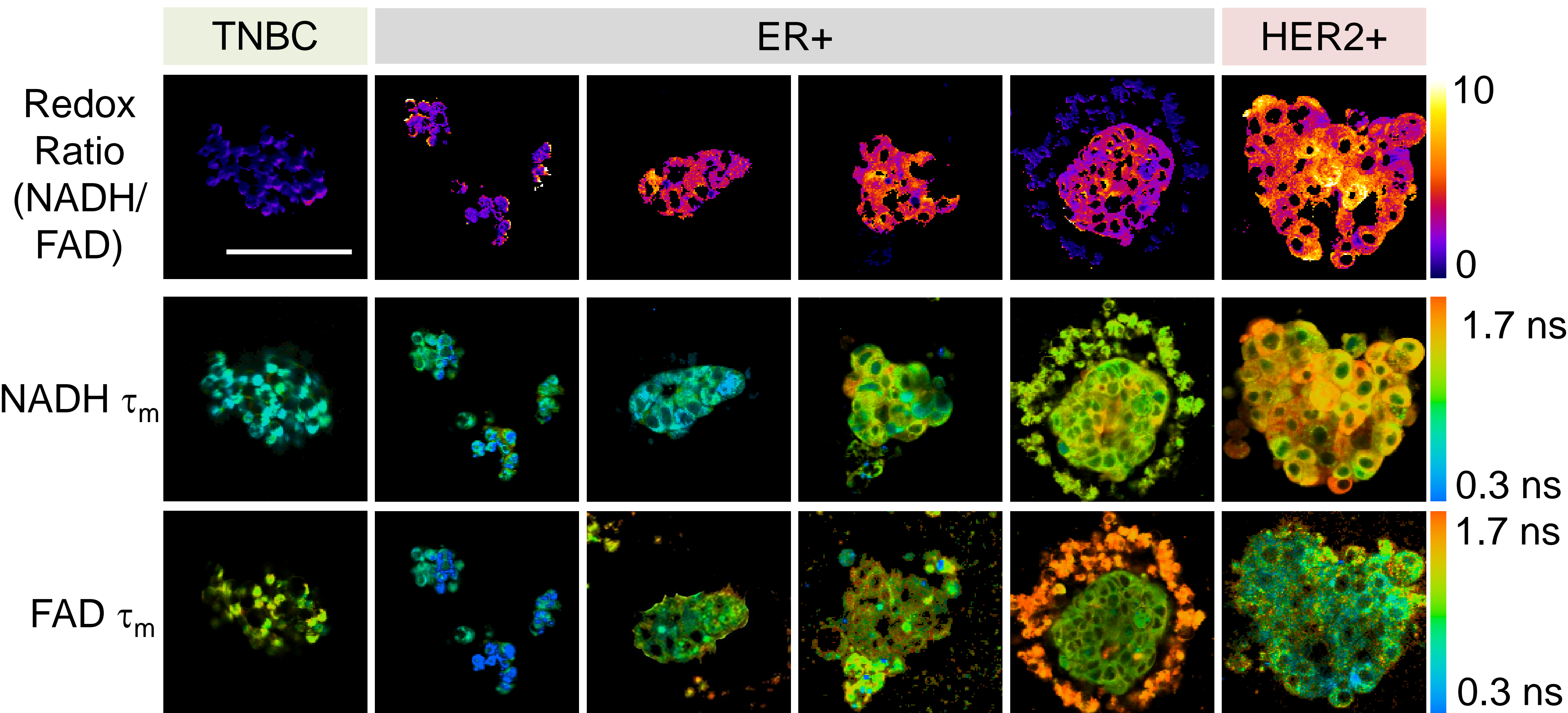


Figure-4 (Skala)

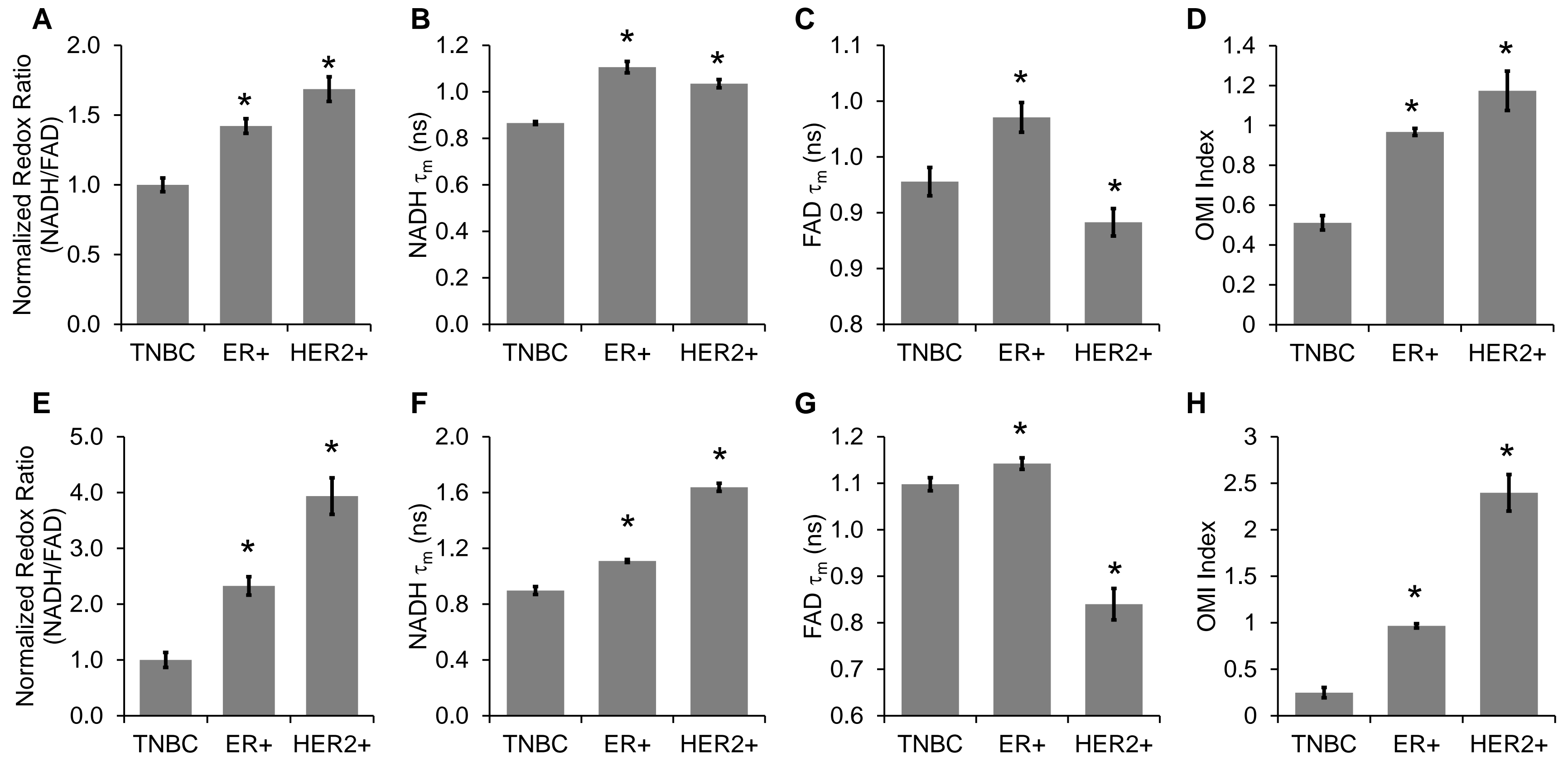


Figure-5 (Skala)

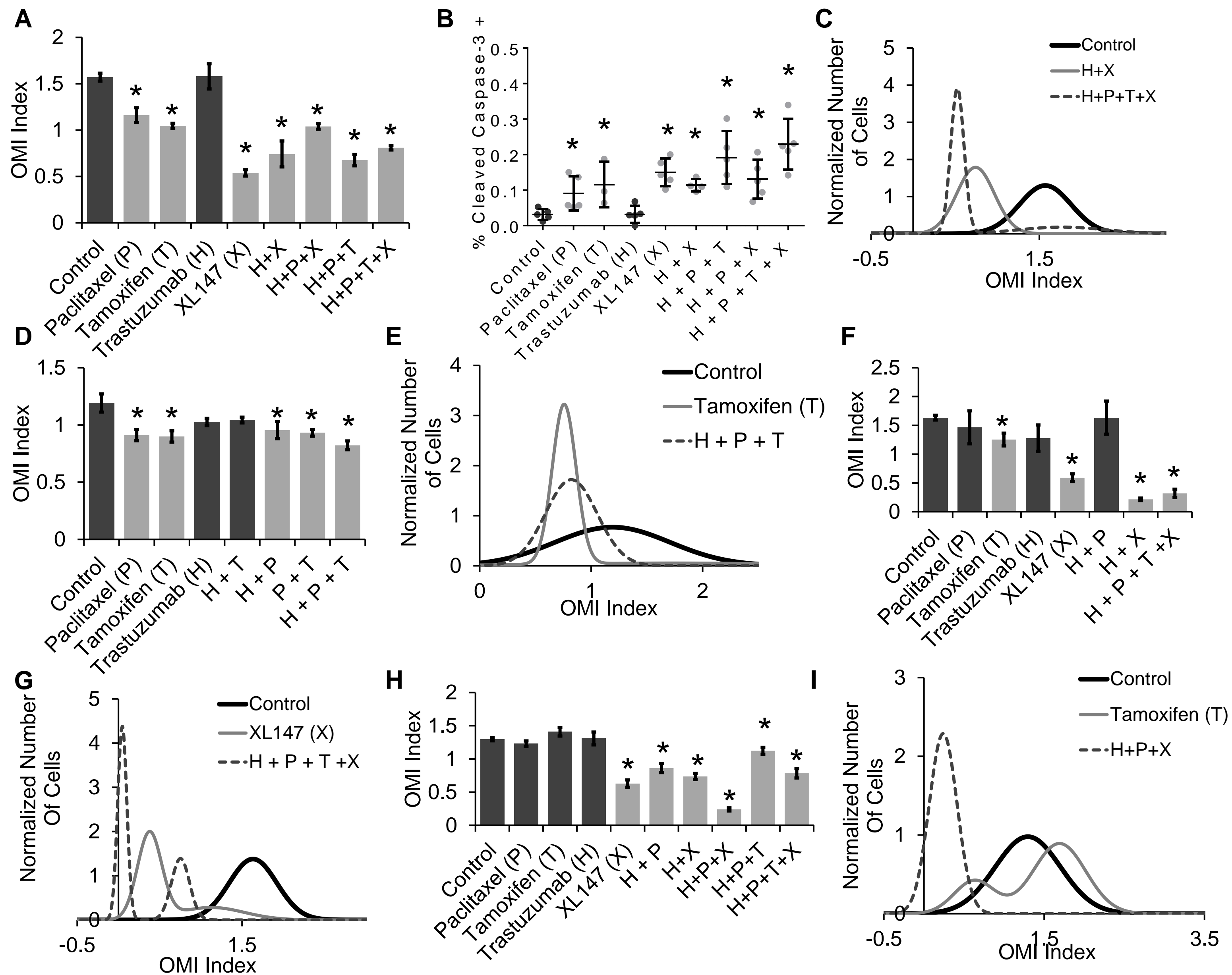
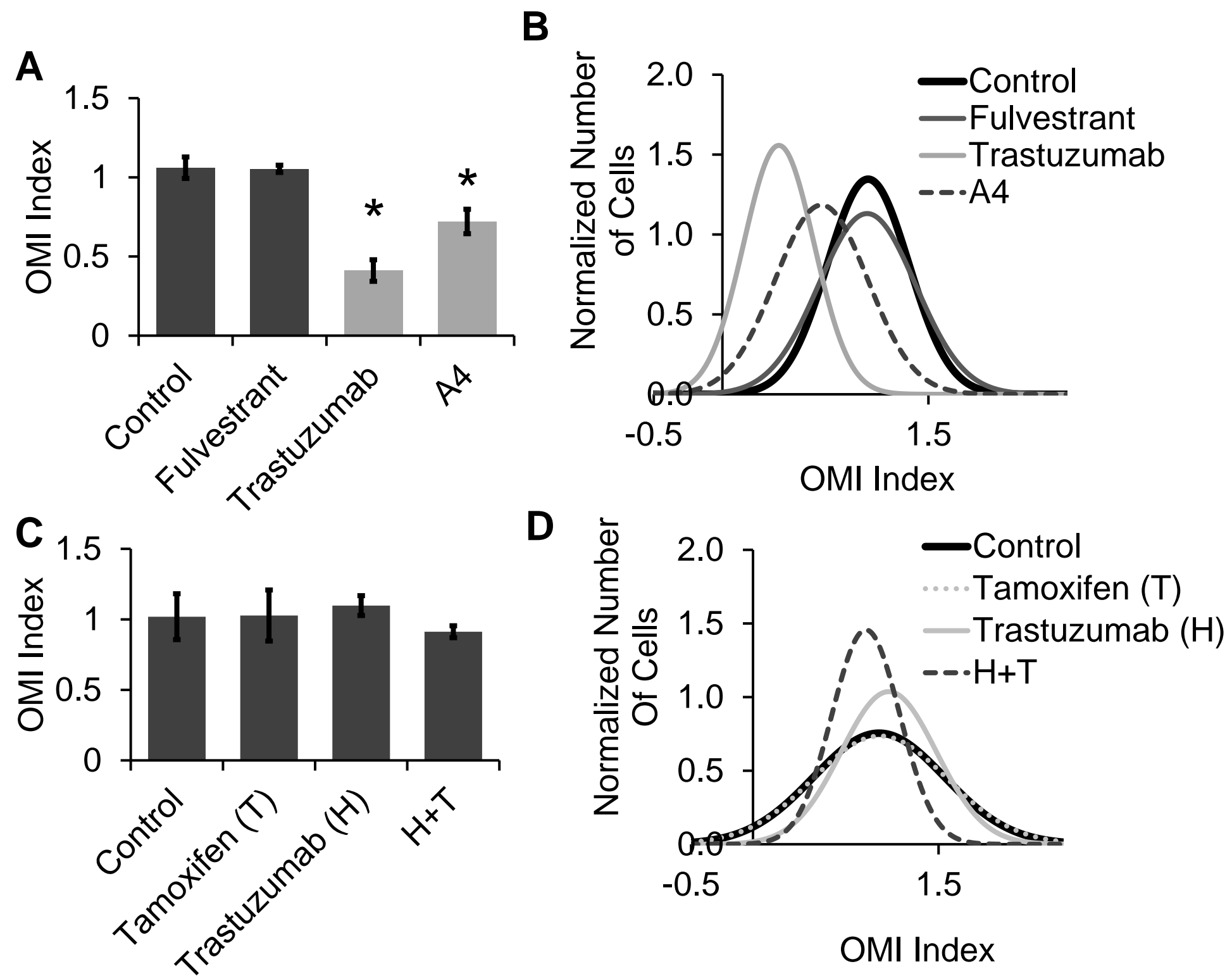


Figure-6 (Skala)



Cancer Research



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Optical Metabolic Imaging Identifies Glycolytic Levels, Subtypes, and Early-Treatment Response in Breast Cancer

Alex J. Walsh¹, Rebecca S. Cook^{2,5}, H. Charles Manning^{1,4}, Donna J. Hicks², Alec Lafontant¹, Carlos L. Arteaga^{2,3,5}, and Melissa C. Skala¹

Abstract

Abnormal cellular metabolism is a hallmark of cancer, yet there is an absence of quantitative methods to dynamically image this powerful cellular function. Optical metabolic imaging (OMI) is a noninvasive, high-resolution, quantitative tool for monitoring cellular metabolism. OMI probes the fluorescence intensities and lifetimes of the autofluorescent metabolic coenzymes reduced NADH and flavin adenine dinucleotide. We confirm that OMI correlates with cellular glycolytic levels across a panel of human breast cell lines using standard assays of cellular rates of glucose uptake and lactate secretion ($P < 0.05$, $r = 0.89$). In addition, OMI resolves differences in the basal metabolic activity of untransformed from malignant breast cells ($P < 0.05$) and between breast cancer subtypes ($P < 0.05$), defined by estrogen receptor and/or HER2 expression or absence. *In vivo* OMI is sensitive to metabolic changes induced by inhibition of HER2 with the antibody trastuzumab (herceptin) in HER2-overexpressing human breast cancer xenografts in mice. This response was confirmed with tumor growth curves and stains for Ki67 and cleaved caspase-3. OMI resolved trastuzumab-induced changes in cellular metabolism *in vivo* as early as 48 hours posttreatment ($P < 0.05$), whereas fluorodeoxyglucose-positron emission tomography did not resolve any changes with trastuzumab up to 12 days posttreatment ($P > 0.05$). In addition, OMI resolved cellular subpopulations of differing response *in vivo* that are critical for investigating drug resistance mechanisms. Importantly, OMI endpoints remained unchanged with trastuzumab treatment in trastuzumab-resistant xenografts ($P > 0.05$). OMI has significant implications for rapid cellular-level assessment of metabolic response to molecular expression and drug action, which would greatly accelerate drug development studies. *Cancer Res*; 73(20); 6164–74. ©2013 AACR.

Introduction

Cellular metabolism produces energy and macromolecules necessary for cell survival. Abnormal metabolism is involved in many of the diseases that cause the greatest burden of morbidity and mortality in the developed world. Many malignant cancer cells maintain high rates of glycolysis in the presence of oxygen (1) and oncogenic transformation is linked with changes in metabolic rates. For example, the HER2 receptor tyrosine kinase, which is amplified in about 20% of invasive breast cancer, potentially activates the phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR pathway, a master regulator of glucose metabolism (2, 3). Patients with HER2 gene-amplified breast cancers present

with more aggressive disease and generally have a poor prognosis (4). HER2 inhibitors such as the antibody trastuzumab (herceptin) provide substantial clinical benefits. However, the action of HER2 inhibitors is limited because of innate and acquired drug resistance (5).

Clinically and in preclinical drug development, there is a need for high-resolution, noninvasive, functional imaging tools to monitor and predict drug efficacy versus lack of efficacy. In cancer research, the primary endpoint of drug efficacy is tumor regression. However, cellular and molecular changes precede changes in tumor size. If these molecular endpoints could be identified and measured, they would provide biomarkers predictive of drug response or drug resistance. Cellular metabolism is particularly sensitive to upstream molecular interventions and therefore may be a powerful biomarker of early-drug response. The HER2 inhibitor trastuzumab, for example, inhibits PI3K-mediated glucose metabolism (6–8). Current preclinical and clinical methodologies to assess metabolic state in tumors *in situ* include fluorodeoxyglucose-positron emission tomography (FDG-PET), immunohistochemical (IHC) assessment of levels of metabolic regulators, and metabolic flux analyses (7, 9–13). Yet each of these techniques fails to capture dynamic changes in metabolic state and poorly reflect sensitivity to drug efficacy (7, 9, 14–18).

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Optical metabolic imaging (OMI) exploits the autofluorescent properties of reduced NADH and flavin adenine dinucleotide (FAD), two metabolic coenzymes. We use multiphoton fluorescence and time-correlated single photon counting to measure the optical redox ratio and fluorescence lifetimes of NADH and FAD in living cells and tissues. The optical redox ratio is the ratio of NADH fluorescence intensity divided by FAD fluorescence intensity (19) and provides a dynamic measure of cellular metabolism (8, 19–21). The fluorescence lifetime, the time a molecule remains in the excited state, is independent of inter- or intrainstrument variability, resolves free and bound protein configurations, and is influenced by preferred protein-binding of the molecules and proximity to quenchers (e.g., oxygen; ref. 22). NADH and FAD each have two-component fluorescence decays. For NADH, the short lifetime (τ_1) corresponds to NADH free in solution, whereas the long lifetime (τ_2) corresponds to protein-bound NADH (23). Conversely, protein-bound FAD corresponds to the short lifetime, whereas free FAD corresponds with the long lifetime (24). The shorter fluorescence lifetimes of both protein-bound FAD and free NADH are due to dynamic quenching by the adenine moiety (22, 25). The mean fluorescence lifetime (τ_m) is the weighted average of the short and long lifetime components, $\tau_m = \alpha_1\tau_1 + \alpha_2\tau_2$, where α_1 and α_2 are the fractional contributions of the short and long lifetimes, respectively.

The images acquired by OMI provide sufficient resolution and contrast to distinguish the cellular and extracellular tissue compartments, as the collagen-enriched ECM generates a strong second harmonic signal that has a lifetime and spectral emission distinct from cellular NADH and FAD (26). Resolution is adequately high to isolate single cells, allowing identification of inflammatory infiltrates in the stroma and tumor epithelia. This single cell-level resolution may be useful for identifying resistant subpopulations of cells that preexist in the tumor and are responsible for cancer relapse.

Because OMI is inexpensive, fast, and directly measures dynamic changes in cellular metabolism that reflect glycolysis, oxidative phosphorylation, and metabolic enzyme microenvironment interactions, we investigated the potential of OMI as a tool for monitoring metabolic response to targeted therapies in human breast cancer cells and xenografts. OMI was validated by measuring metabolic inhibition by cyanide, and in comparison with standard assays of glycolytic metabolism. The sensitivity of OMI to breast cancer subtypes was confirmed. Finally, the OMI-measured response of mouse xenograft models treated with trastuzumab was compared with FDG-PET, IHC, and tumor size measurements. This work represents a significant advancement in the tools available to study cellular metabolism and tumor response to treatment in living systems.

Materials and Methods

Fluorescence lifetime instrumentation

A custom built, commercial multiphoton fluorescence microscope (Prairie Technologies) was used to acquire fluorescence images. A 40X water-immersion objective (1.15 NA) or a 40X oil-immersion objective (1.3 NA) coupled the excitation

and emitted light through an inverted microscope (TiE, Nikon). A titanium:sapphire laser (Coherent Inc.) was tuned to 750 nm for excitation of NADH and 890 nm for FAD excitation. The average laser power was 7.5 to 7.8 mW for NADH and 8.4–to 8.6 mW for FAD. A pixel dwell time of 4.8 μ s was used. A GaAsP PMT (H7422P-40, Hamamatsu) detected emitted photons. A 400 to 480 nm bandpass filter isolated NADH fluorescence. A 500 nm high pass dichroic mirror and a 500 to 600 nm bandpass filter isolated FAD fluorescence.

Fluorescence lifetime images were acquired using time correlated single photon counting (TCSPC) electronics (SPC-150, Becker and Hickl). TCSPC uses a fast detector PMT to measure the time between a laser pulse and fluorescence event. Each image of 256×256 pixels was acquired using an integration time of 60 seconds. No change in the photon count rate was observed, ensuring that photobleaching did not occur. The instrument response function (measured from the second harmonic generated signal of urea crystals excited at 900 nm) full width at half maximum was measured to be 260 ps. The single-component fluorescence lifetime of a fluorescent bead (Polysciences Inc.) was measured daily. The measured fluorescence lifetime of the bead was 2.1 ± 0.08 ns ($n = 18$), which is consistent with published studies (20, 27).

Cell culture

All cell lines were acquired from the American Type Culture Collection except the HR6 cell line (28), which was provided by the Arteaga Laboratory. The noncancerous mammary epithelium cell line, MCF10A, was cultured in MEBM (Lonza) supplemented with cholera toxin, penicillin: streptomycin, bovine pituitary extract, hydrocortisone, insulin, and human epidermal growth factor. All malignant cell lines were grown in DMEM (Invitrogen) with 10% FBS and 1% penicillin: streptomycin. The growth media for the HR6 cell line was further enhanced with 25 μ g/mL trastuzumab (Vanderbilt Pharmacy). For fluorescence imaging, cells were plated at a density of 10^6 cells per 35 mm glass-bottom imaging dish (MatTek Corp.) 48 hours before imaging.

The MCF10A cell line was used as a daily fluorescence standard for the redox ratio and imaged each day measurements were acquired. All other cell lines were imaged on at least two different days. A total of 18 different locations were imaged for each cell line (58 for MCF10A cells) from six different dishes (three images were acquired from each dish, see Supplementary Table S1).

Cyanide experiment

NADH and FAD fluorescence lifetime images of three locations of three dishes were acquired. Media of two of the MCF10A dishes was removed and replaced with cyanide supplemented MCF10A growth media (4 mmol/L NaCN, Sigma). The cells were allowed 5 minutes for the cyanide to react and post-cyanide NADH and FAD fluorescence images were acquired from three unique locations from each dish.

Trastuzumab perturbation

The effect of HER2 inhibition by trastuzumab was tested in HER2-overexpressing cells. The cells were plated at a density of

10^6 cells per imaging dish, 48 hours before imaging. At 24 hours before imaging, the growth media was exchanged for growth media containing 25 $\mu\text{g/mL}$ trastuzumab. This dose of trastuzumab, 25 $\mu\text{g/mL}$, was chosen to mimic therapeutic drug dosage in patients (29).

Mouse xenografts

This study was approved by the Vanderbilt University Animal Care and Use Committee and meets the NIH guidelines for animal welfare. MDA-MB-361 cells (10^6), BT474 cells (10^8), or HR6 cells (10^8) in 100 μL Matrigel were injected in the inguinal mammary fat pads of female athymic nude mice (J:NU; Jackson Laboratories). Tumors were allowed to grow to approximately 150 mm^3 . Tumor-bearing mice were treated with trastuzumab (Vanderbilt University Medical Center pharmacy) or control human immunoglobulin G (IgG) 10 mg/kg twice weekly for two weeks. This dose of trastuzumab was chosen to mimic therapeutic drug dosage in patients (30).

OMI xenograft imaging

Isoflurane-anesthetized mice were used for vital imaging, by removing the skin overlying the tumor, overlaying the tumor with a coverslip, and placing the mouse on the microscope stage. NADH and FAD fluorescence lifetime images of three different tumor locations were acquired each day. After imaging, mice were humanely euthanized while under anesthesia. Each OMI group contained 3 mice, each with 2 tumors for a total n of 6 tumors at each time point.

FDG-PET imaging

The FDG-PET protocol follows published methods (7, 31, 32). The mice were fasted overnight and allowed to acclimate to the PET facility for 1 hour on a warm water pad. A single retroorbital injection of approximately 200 μCi (100 μL) of [^{18}F]FDG was administered. Following a 40-minute distribution period, 20-minute static PET scans were collected on a Concorde Microsystems microPET Focus 220 (Siemens), whereas mice were anesthetized with isoflurane. PET images were reconstructed using the ordered subsets expectation maximization algorithm (33). FDG-uptake values were obtained by isolating the uptake of each tumor volume and correcting for the injected dose. Each FDG-PET group contained 5 mice, each with 2 tumors for a total n of 10 tumors.

Quantification of the optical redox ratio

The optical redox ratio was computed from the NADH and FAD fluorescence lifetime data. The photons detected at each pixel in an image were integrated over time to compute the sum of photons per pixel. The total number of NADH photons was divided by the total number of FAD photons at each pixel to create a redox ratio image (MATLAB, MathWorks). The redox ratio image was thresholded to remove background and nuclear fluorescence and the average redox ratio for the remaining cell cytoplasm was computed. This approach has been confirmed to be consistent with redox ratios obtained with steady-state detection (8, 21).

Quantification of fluorescence lifetime components

For each image, a threshold was selected to eliminate background and nuclear fluorescence (SPCImage, Becker and Hickl). A binning of nine surrounding pixels was used. Then, the fluorescence lifetime components were computed for each pixel by deconvolving the measured system response and fitting the resulting exponential decay to a two-component model, $I(t) = \alpha_1 \exp^{-t/\tau_1} + \alpha_2 \exp^{-t/\tau_2} + C$, where $I(t)$ is the fluorescence intensity at time t after the laser excitation pulse, α_1 and α_2 are the fractional contributions of the short and long lifetime components, respectively (i.e., $\alpha_1 + \alpha_2 = 1$), τ_1 and τ_2 are the fluorescence lifetimes of the short and long lifetime components, and C accounts for background light. A two-component decay was used to represent the lifetimes of the free and bound configurations of NADH and FAD (20, 23, 24). The average lifetime component values and a mean fluorescence lifetime ($\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2$) for each image were computed in MATLAB.

Statistical analysis

A rank sum test of means was used to test for significant differences due to cyanide. A Bonferroni correction for multiple-comparisons was used on rank sum tests of means of the metabolic values from the panel of cell lines. A rank sum test of means was used to identify significant differences when cell lines were treated with trastuzumab and to find differences in the *in vivo* xenograft experiments. A Student t test of means tested for significantly different FDG-uptake values between control and trastuzumab-treated xenografts. For all statistical tests, an $\alpha_{\text{significance}}$ level of 0.05 was used and the test was assumed to be two-way.

Spearman rank correlation coefficient was used to identify correlations. Both a correlation coefficient (r) and a P value were computed. An α level of less than 0.05 signified significance. Scatterplots of the significant correlations confirmed that the correlation was due to data trends and not a single outlier.

Computation of intra- and intercellular variation

Intercellular variation was visualized by histogram representation of the mean metabolic measure (optical redox ratio, NADH τ_m , or FAD τ_m) for all cells. The histogram was fit to one, two, and three component Gaussian curves to determine the number of modes within the data. The fit with the lowest Akaike information criterion was selected to represent the probability density function of the histogram (34). Intracellular variation was computed as the average coefficient of variation (SD divided by mean) for each cell and averaged over all cells.

Percentage of mitotic cells

The percentage of proliferating cells was measured by flow cytometry. Cells were plated at a density of 10^6 cells per 35 mm dish. After 48 hours, the cells were labeled with Phospho-Histone H3 (Ser10) antibody (Cell Signaling Technology) and a secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) enabled detection of labeled cells by flow cytometry.

Glycolytic index

Media glucose and lactate concentrations were measured using standard assay kits [Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen) and L-Lactate Assay Kit (Eton Bioscience Inc.)]. Concentrations of glucose and lactate in the cell growth media were determined at the time of plating (0 hour) and at the time of imaging (48 hour). The "glycolytic index" was computed as the moles of glucose consumed within 48 hours divided by the moles of lactate produced in 48 hours.

Histologic analysis

Tumors were collected and placed in buffered formalin, paraffin embedded, sliced, and stained with hematoxylin and eosin stain. Additional slides were stained for Ki-67 and cleaved caspase-3. Staining protocols were verified in positive control samples. The percentage of positively stained cells was quantified from five fields of view from three tumors in each group.

Results

Figure 1A shows the relationship between HER2 and the fluorescent molecules NADH and FAD. HER2 activation drives an increase in glycolysis, which generates NADH. The pyruvate produced in glycolysis can enter the mitochondria as a reactant in oxidative phosphorylation. Oxidative phosphorylation consumes NADH and produces FAD. A net gain of NADH relative to FAD is observed with HER2 activation due to a relative increase in glycolysis.

Validation

Chemical inhibition of oxidative phosphorylation affects the relative fluorescence intensities of NADH and FAD in a cell (35), so this perturbation was used to validate the optical imaging approach (Fig. 1B–D, Supplementary Fig. S1). Cyanide (which disrupts the electron transport chain) induced the predicted trends (23, 27): an increase in the optical redox ratio and a decrease in NADH τ_m . Unreported until now, FAD τ_m increased with cyanide treatment.

Metabolic profiling of breast cancer cells

High-resolution images (Fig. 2) allowed visualization of cellular morphology and cell-to-cell variability of the optical redox ratio and NADH and FAD fluorescence lifetimes for human breast cell lines (Supplementary Table S1). Cellular fluorescence localized to the cytoplasm of cells. ER⁺/HER2[−] cells displayed increased redox ratios over that seen in triple-negative cells ($P < 0.001$; Fig. 3A), and the greatest redox ratios were measured in HER2 gene-amplified cells ($P < 0.001$). To account for any differences in cellular proliferation rates among the cell lines and show that the redox ratio is not a reporter of cellular proliferation (Supplementary Fig. S2A), we normalized the redox ratio to the percentage mitotic cells (Supplementary Fig. S2B) and found similar trends, increased redox ratio/percentage mitotic cells in ER⁺ cells and the greatest redox ratio/percentage mitotic cells in HER2⁺ cells.

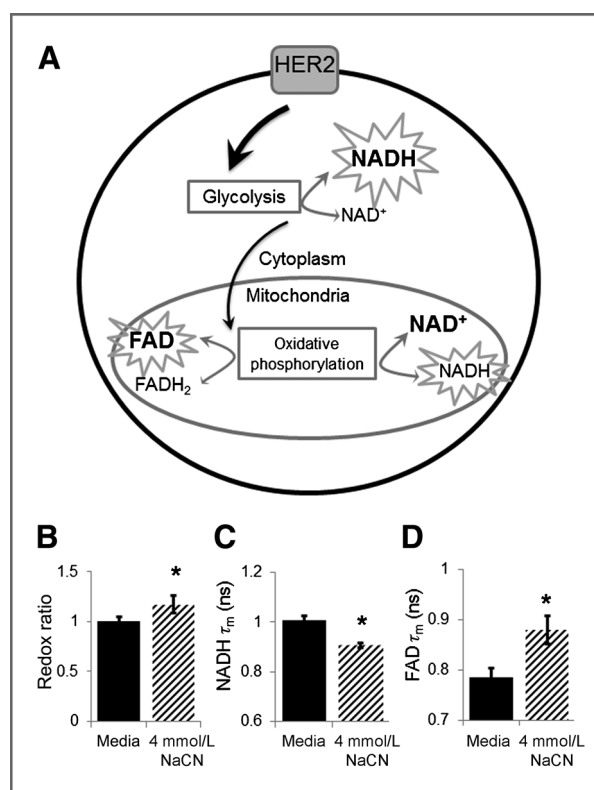


Figure 1. A, graphical representation of the relationship between HER2, NADH, and FAD. HER2 activation drives an increase in glycolysis, which produces NADH. The pyruvate generated in glycolysis can enter the mitochondria as a reactant in oxidative phosphorylation. Oxidative phosphorylation consumes NADH and produces FAD. A net gain of NADH relative to FAD is observed with HER2 activation due to a relative increase in glycolysis. Inhibition of HER2 by trastuzumab reduces cellular glycolysis rates, resulting in a decrease of cellular NADH relative to FAD. NADH and FAD are highlighted as the fluorescent molecules in this diagram and molecules in bold indicate the net direction of the reaction. Optical redox ratio (B), NADH τ_m (C), and FAD τ_m (D) of MCF10A (nonmalignant) cells before ($n = 9$) and after ($n = 6$) treatment with 4 mmol/L NaCN. τ_m is the mean lifetime ($\tau_m = \tau_1 \alpha_1 + \tau_2 \alpha_2$). *, $P < 0.05$.

NADH τ_m of HER2⁺ and ER⁺ cells were increased over that measured in nonmalignant cells ($P < 0.001$; Fig. 3B), but were statistically similar to each other ($P = 0.5$). Triple-negative cells exhibited the shortest NADH τ_m . Reduced-free and protein-bound NADH lifetimes (τ_1 and τ_2) were observed in the triple-negative and ER⁺ cells (Supplementary Fig. S3A and S3B). The portion of free NADH (α_1) was decreased in the HER2⁺ cells compared with the nonmalignant cells (Supplementary Fig. S3C). Compared with nonmalignant cells, FAD τ_m was increased in all malignant cells ($P < 0.001$), with the longest FAD τ_m observed in ER⁺/HER2[−] cells ($P < 0.001$; Fig. 3C). Increased FAD τ_1 and τ_2 values, as well as a reduced α_1 (contribution of bound FAD) contributed to the increased FAD τ_m observed in the malignant cells (Supplementary Fig. S3D–S3F). A scatterplot of NADH τ_m versus redox ratio/percentage mitotic cells (Fig. 3D) allowed accurate clustering of data-points of nonmalignant, triple-negative, ER⁺, and HER2⁺ cells. Scatterplots of FAD τ_m versus redox ratio and

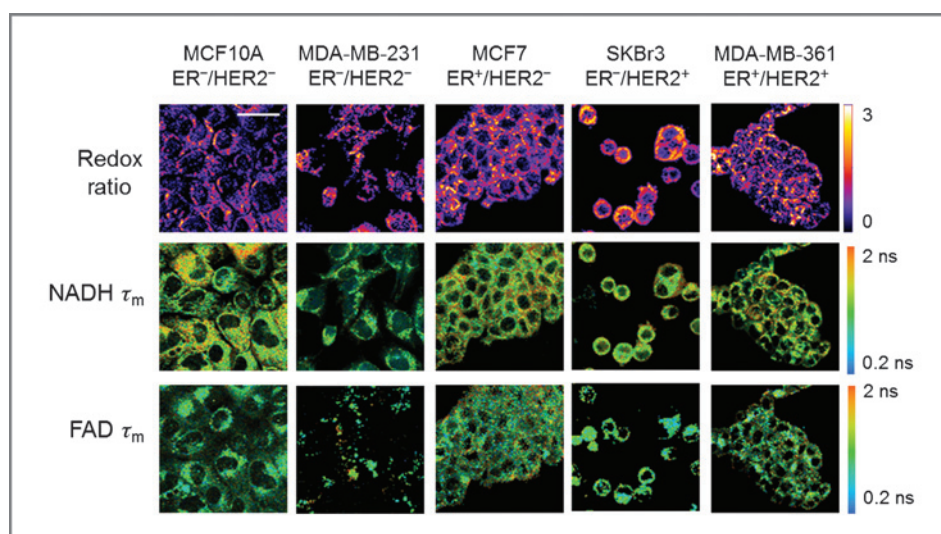


Figure 2. Representative images of the optical redox ratio (NADH/FAD; first row), NADH τ_m (second row), and FAD τ_m (third row) of MCF10A (nonmalignant) and malignant breast cells. Scale bar, 30 μ m. The redox ratio is normalized to the mean daily MCF10A mean redox ratio as a daily fluorescence standard. τ_m is the mean lifetime ($\tau_m = \tau_1 \alpha_1 + \tau_2 \alpha_2$). The ER and HER2 status of each cell line is provided under its name.

NADH τ_m versus FAD τ_m allow separation of triple-negative and nonmalignant cells (Supplementary Fig. S4).

We compared OMI with cellular rates of glucose uptake and lactate secretion or the "glycolytic index." A Spearman rank correlation coefficient (r) of 0.89 ($P < 0.05$) defined a positive correlation between the optical redox ratio/percentage mitotic cells and the glycolytic index (Fig. 3E). Neither NADH τ_m nor

FAD τ_m correlated with the glycolytic index or the redox ratio (Supplementary Table S2).

Trastuzumab response *in vitro*

The effect of trastuzumab on cellular metabolism was investigated in three HER2-overexpressing cell lines: BT474 cells that are responsive to trastuzumab, MDA-MB-361

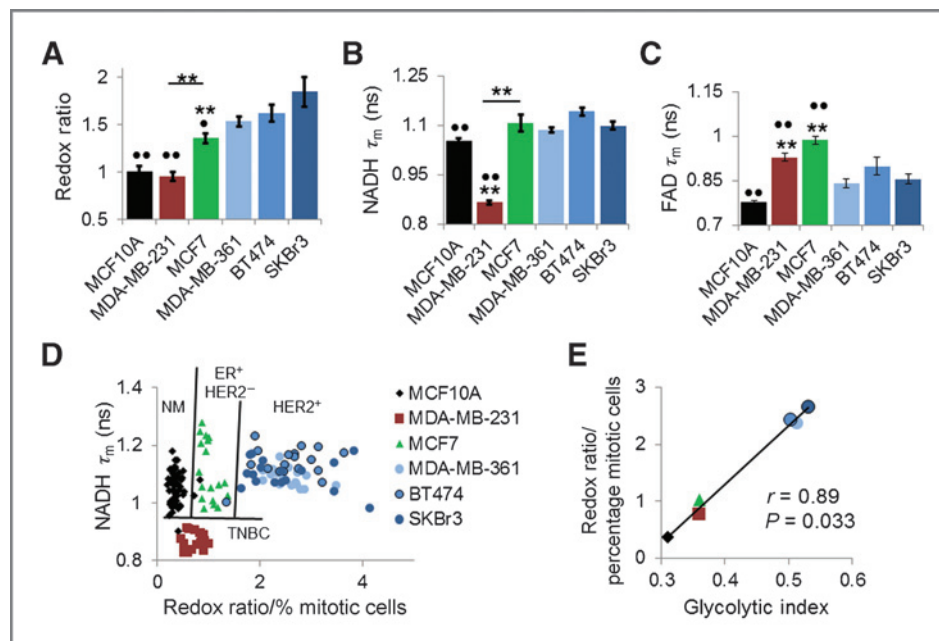


Figure 3. A, the optical redox ratio (mean \pm SE) is increased in malignant cells with ER overexpression (MCF7, green) and is further increased in cells with HER2 overexpression (MDA-MB-361, BT474, and SKBr3; blue). B, the NADH τ_m (mean \pm SE) is decreased in triple-negative cells (MDA-MB-231, red) relative to nonmalignant, ER⁺, and HER2⁺ cells. The NADH τ_m is increased in HER2⁺ cells relative to nonmalignant cells. C, FAD τ_m (mean \pm SE) is increased in malignant cells relative to nonmalignant cells. D, a scatterplot of NADH τ_m versus redox ratio/percentage mitotic cells provides visual separation of the different molecular subtypes of breast cancer [nonmalignant (NM), ER⁺/HER2⁻, HER2⁺, and triple-negative (TNBC)]. E, correlation between redox ratio/percentage mitotic cells and glycolytic index. Unless indicated with a line, asterisks (*) indicate statistically significant differences with the MCF10A (nonmalignant) cells and bullets (•) indicate statistically significant differences with the HER2⁺ cells grouped together. **, $P < 0.001$. $n = 18$ for all malignant cell lines and $n = 58$ for MCF10A.

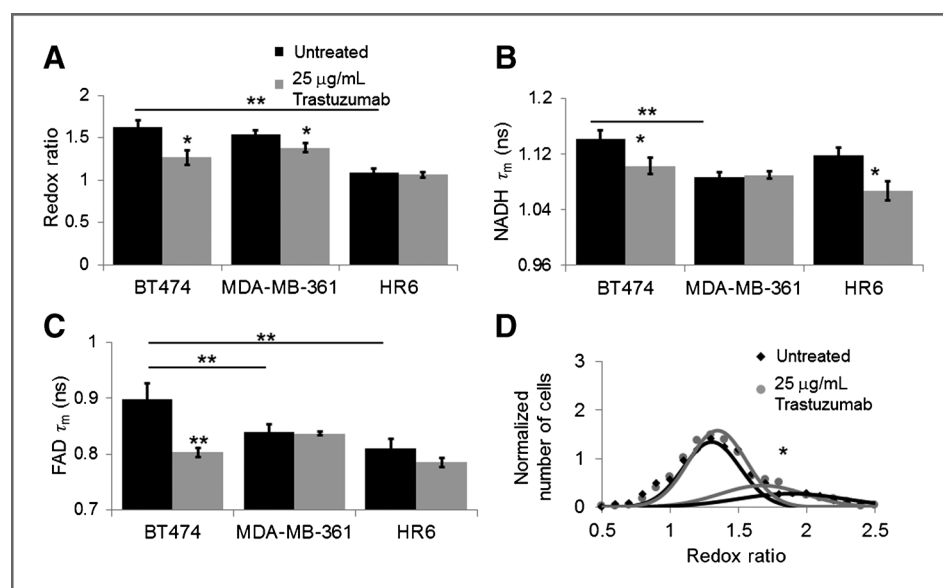


Figure 4. A, the redox ratio (mean \pm SE) decreases with trastuzumab treatment in responsive (BT474) and partially responsive (MDA-MB-361) cells but remains unchanged in cells with acquired resistance to trastuzumab (HR6). B, NADH τ_m (mean \pm SE; $\tau_m = \tau_1^* \alpha_1 + \tau_2^* \alpha_2$) is shorter in trastuzumab-treated responsive cells (BT474) and cells with acquired resistance (HR6), but unchanged in cells with poor response (MDA-MB-361). C, FAD τ_m (mean \pm SE) is shorter in trastuzumab-treated BT474 but unchanged MDA-MB-361 and HR6 cells. *, significance between control and treated, unless otherwise indicated. D, distribution density modeling (black line, fit of untreated cell histogram; gray line, fit of trastuzumab-treated cells) of the redox ratio on a cell-by-cell basis reveals two distinct populations of MDA-MB-361 cells for the redox ratio, with 70% and 30% of cells in the majority and minority populations, respectively, for both control and treated cells. There is a significant decrease ($P < 0.05$) in the mean of the minority population of treated cells (but not in the majority population), suggesting trastuzumab response in a subpopulation of cells. **, $P < 0.001$; *, $P < 0.05$. $n = 18$ images for control and treated for all cell lines.

cells that partially respond to trastuzumab, and HR6 cells, which were derived from a BT474 xenograft that acquired resistance to trastuzumab *in vivo* (28). The redox ratio ($P < 0.05$), NADH τ_m ($P < 0.05$), and FAD τ_m ($P < 0.001$) of BT474 cells decreased upon trastuzumab treatment for 24 hours (Fig. 4A–C). Lifetime component analysis showed significant increases in the portions of free NADH (α_1) and bound FAD (α_1) of BT474 cells treated with trastuzumab (Supplementary Fig. S5). Similarly, the redox ratio of the MDA-MB-361 cells decreased ($P < 0.05$) with trastuzumab treatment (Fig. 4A). The high-resolution OMI enabled analysis of cellular heterogeneity in response to trastuzumab (Fig. 4D, Supplementary Fig. S6). Distribution modeling of cellular redox ratios of MDA-MB-361 cells revealed two subpopulations characterized by differing redox ratios (Fig. 4D). The mean redox ratio of the first population, representing 70% of cells, showed no change (1.34 to 1.31) upon trastuzumab treatment ($P = 0.07$), suggesting that trastuzumab did not affect metabolic processes in the majority of MDA-MB-361 cells. However, 30% of the cells responded to trastuzumab with a mean redox ratio that decreased from 1.89 to 1.65 ($P < 0.001$).

HR6 cells are a trastuzumab-resistant BT474-derived subline that retains HER2 overexpression. Previous studies have shown maintenance of HER2 overexpression and PI3K/Akt signaling in trastuzumab-treated HR6 cells (28). Consistent with this observation, the redox ratio remained unchanged upon treatment with trastuzumab, as was FAD τ_m . NADH τ_m decreased in HR6 cells upon treatment with the antibody

($P < 0.05$; Fig. 4A–C). An increased portion of free NADH (α_1) contributed to the decreased NADH τ_m observed in the HR6 cells (Supplementary Fig. S5).

In vivo xenografts

To verify the OMI response observed in cultured cells, we conducted OMI on HER2-overexpressing xenografts and compared these findings with tumor size, IHC, and FDG-PET measurements. Established BT474 xenografts treated with control IgG continued to grow throughout the course of the experiment while the trastuzumab-treated tumors decreased in size (Fig. 5A). IHC staining confirmed lower rates of proliferation and higher rates of apoptosis in the trastuzumab-treated group (Fig. 5B and C). A representative FDG-PET image displays the location of the tumors in the mammary fat near the hind limbs and shows increased FDG-uptake in the tumors compared with the surrounding tissue (Fig. 5D). FDG-uptake increased in the control mice between days 2 and 12 post-treatment; however, no difference was observed between control and trastuzumab-treated tumors at any time point (day 2, 5, or 12 posttreatment; Fig. 5E). OMI imaging of an identical cohort of tumors allowed cellular-level visualization of metabolism (Fig. 5F) and resolved metabolic differences in the redox ratio (Fig. 5G), NADH τ_m (Fig. 5H, Supplementary Fig. S7A–S7C), and FAD τ_m (Fig. 5I, Supplementary Fig. S7) between control and trastuzumab-responding tumors as early as 2 days after the first dose of trastuzumab.

Tumor size measurements of the HR6 xenografts showed similar growth of both control and trastuzumab-treated HR6

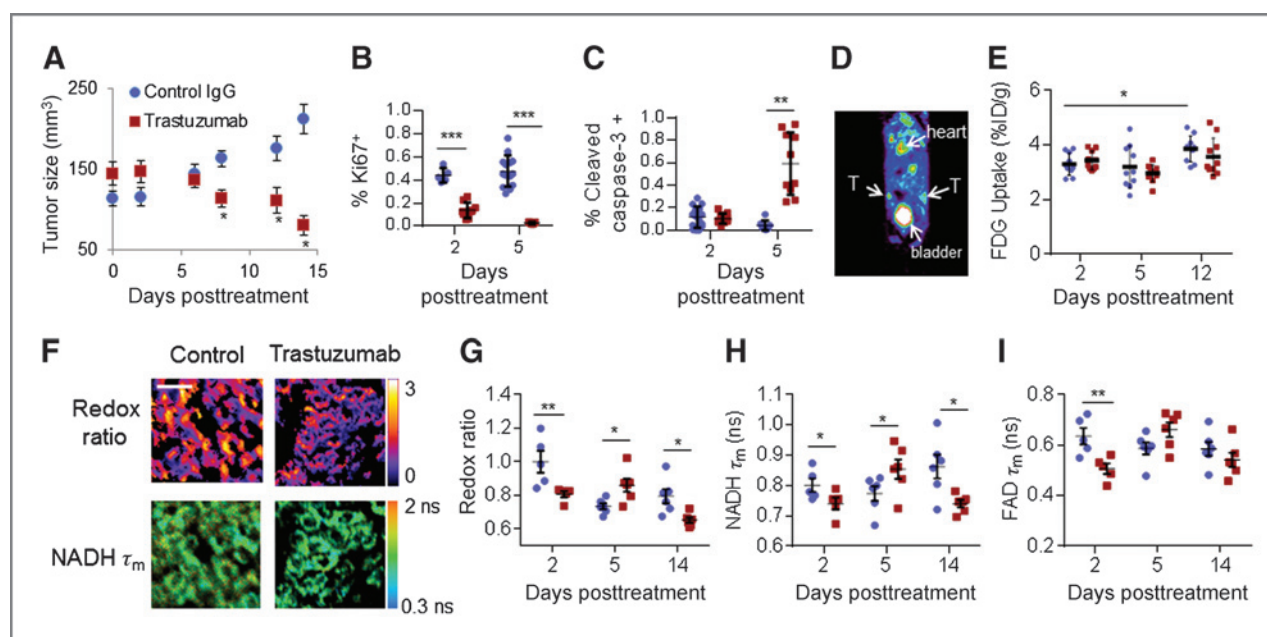


Figure 5. A, BT474 tumors treated with trastuzumab (10 mg/kg, 2× weekly) decrease in size (mean ± SE) compared with control IgG-treated tumors. Trastuzumab versus control at each time point. B, Ki67 staining shows reduced proliferation in trastuzumab-treated tumors. C, cleaved caspase-3 staining shows increased apoptosis in trastuzumab-treated tumors at day 5. D, representative FDG-PET image (T, tumor). E, FDG-uptake increases in control tumors at day 12 compared with control tumors at day 2. No significant difference in FDG-uptake between trastuzumab-treated and control tumors is observed. $n = 10$. F, representative OMI images. Scale bar, 50 μ m. G, decreased redox ratio, NADH τ_m (H), and FAD τ_m (I) are observed in trastuzumab-treated tumors at 2 days after trastuzumab-treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 6$ tumors.

tumors (Fig. 6A). IHC confirmed that the trastuzumab-treated HR6 tumors retain proliferative capabilities and express similar rates of apoptosis compared with control-treated tumors

(Fig. 6B and C). A representative FDG-PET image of the HR6 tumors shows increased FDG-uptake by the tumor compared with the surrounding tissue (Fig. 6D). No change in FDG-PET

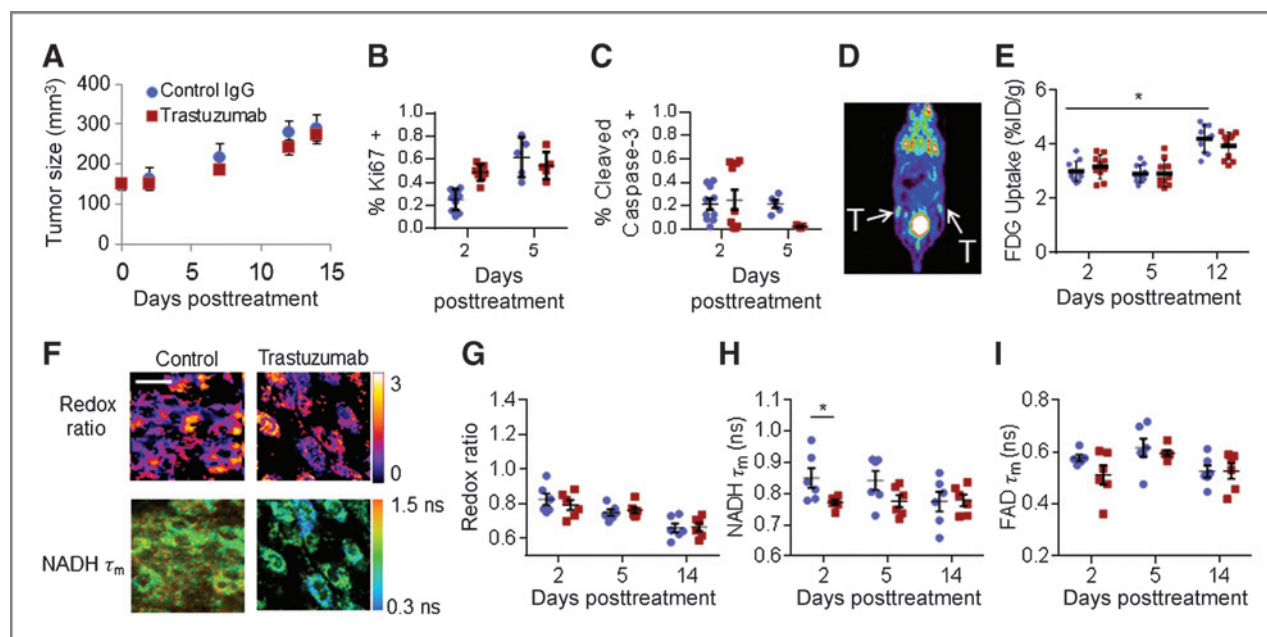


Figure 6. A, HR6 tumor size (mean ± SE) treated with trastuzumab (10 mg/kg, 2× weekly) compared with control IgG-treated tumors. B, Ki67 staining of HR6 control IgG and trastuzumab-treated tumors. C, cleaved caspase-3 staining of HR6 control IgG and trastuzumab-treated tumors. D, representative FDG-PET image (T, tumor). E, FDG-uptake increases in control tumors at day 12 compared with control tumors at day 2, *, $P < 0.05$. No significant difference in FDG-uptake between trastuzumab-treated and control tumors is observed at any time. $n = 10$. F, representative OMI images; scale bar, 50 μ m. G, redox ratio, NADH τ_m (H), and FAD τ_m (I) of control and trastuzumab-treated tumors. *, $P < 0.05$; $n = 6$ tumors.

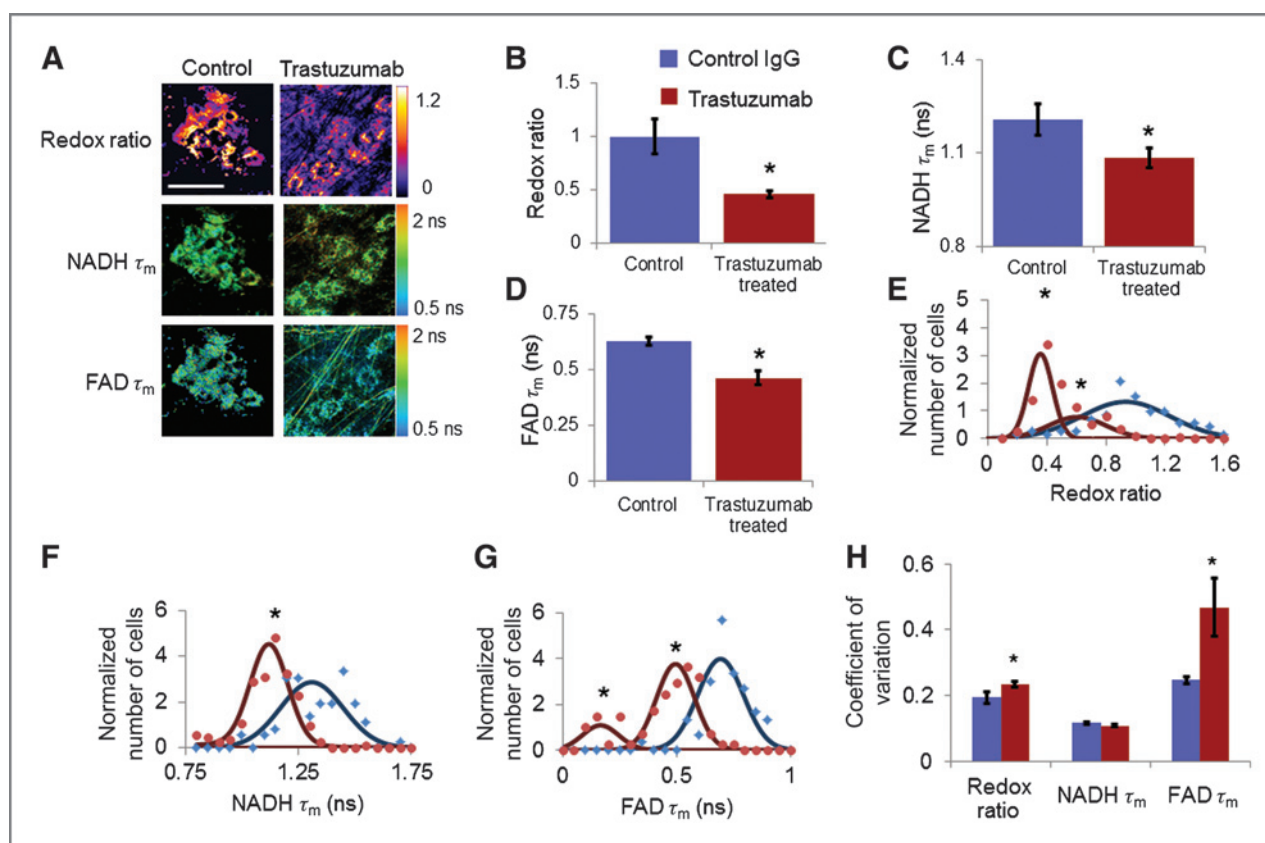


Figure 7. A, representative images of the optical redox ratio (first row), NADH τ_m (second row), and FAD τ_m (third row) of untreated control and trastuzumab-treated (10 μ g/kg; 48 hours) MDA-MB-361 mouse xenograft tumors. Fibrillar structures are due to collagen second harmonic generation. Scale bar, 50 μ m. B–D, mean \pm SE on a per image basis. E–G, distribution density modeling on a cell-by-cell basis in control and trastuzumab-treated tumors. B, the optical redox ratio is reduced in trastuzumab-treated tumors compared with the control tumors. C, NADH τ_m is reduced in the trastuzumab-treated tumors compared with the control tumors. D, FAD τ_m is reduced in trastuzumab-treated tumors compared with the control tumors. E–G, distribution density modeling reveals two distinct populations of trastuzumab-treated cells for the redox ratio (E, maroon line) and FAD τ_m (G, maroon line) measurements, indicating heterogeneous cellular metabolic response to treatment. E–G, blue lines, all control populations and the trastuzumab-treated NADH τ_m population (F, maroon line) are unimodal. H, the increased coefficient of variation in the redox ratio and FAD τ_m of the trastuzumab-treated xenografts indicates increased intracellular variation in metabolism. *, $P < 0.05$.

was observed between control and trastuzumab-treated tumors at any time point; however, the FDG-uptake was increased in the control mice at 12 days compared with 2 days (Fig. 6E). No difference was observed in the redox ratio between control and treated HR6 tumors at any time point (Fig. 6F and G). NADH τ_m decreased in the HR6-treated tumors relative to controls at 2 days posttreatment, but was similar to controls at days 5 and 14 (Fig. 6H, Supplementary Fig. S8). No change in FAD τ_m was observed at any time point (Fig. 6I, Supplementary Fig. S8).

The redox ratio ($P < 0.05$), NADH τ_m ($P < 0.05$), and FAD τ_m ($P < 0.05$) decreased in trastuzumab-treated MDA-MB-361 xenografts as compared with IgG-treated controls (Fig. 7A–D; Supplementary Fig. S9) 2 days after treatment. Modeling of the optical redox ratio and FAD τ_m values on a cell-by-cell basis (intercell variability) identified two subpopulations of tumor cells in trastuzumab-treated animals, whereas an unimodal cellular distribution was identified for NADH τ_m (Fig. 7E–G). High-resolution analysis revealed that intracellular variability in redox ratios and FAD τ_m increased

($P < 0.05$) in trastuzumab-treated samples (Fig. 7H). These results fully characterize OMI and show its potential for monitoring early-drug response in cell culture and mouse xenografts on a single-cell level.

Discussion

OMI is advantageous for live cell and animal imaging due to several features, including direct and dynamic assessment of cellular metabolism *in vivo*, cellular and subcellular resolution imaging capabilities, rapid acquisition, low-cost, use of intrinsic signals (no contrast agents, no radioactivity), and high sensitivity to metabolic changes. Our results have significant implications for rapid assessment of drug action in preclinical models, which would greatly accelerate drug development studies.

These results are the first to correlate OMI with a standard assay of metabolic behavior. Although the optical redox ratio is inferred from theory as the relative rates of glycolysis and oxidative phosphorylation within cells, we confirm a strong

positive correlation ($r = 0.89$, $P = 0.03$) with a standard measure of cellular glycolytic levels (Fig. 3). In contrast, NADH τ_m and FAD τ_m are not correlated with the glycolytic index (Supplementary Table S2). The proportion of free NADH (α_1) has been interpreted as an analog to the optical redox ratio (27); however, we did not find a correlation between the optical redox ratio and NADH α_1 (Supplementary Table S2). Given the physical nature of these fluorescence lifetime measurements, which are sensitive to protein-binding, relative fractions of free and bound components, and proximity to quenchers, it is not surprising that they are sensitive to more cellular processes than just glycolysis. For example, changes in the distribution of NADH and FAD enzyme-binding sites associated with preferred metabolic pathways in breast cancer subtypes may be responsible for the changes in protein-bound lifetimes between cell lines (36). The changes in the lifetimes of the free components of NADH and FAD may reflect changes in dynamic quenching (22). Taken together, the data (Fig. 3, Supplementary Table S2) indicate that the redox ratio, NADH, and FAD lifetimes provide independent measures of cellular metabolism and the molecular microenvironment. The varied dynamics of these endpoints due to HER2 inhibition suggest that all three OMI endpoints provide added value when measured together.

We used these tools to differentiate breast cancer cells by subtype, defined by ER, and/or HER2 expression or absence (Fig. 3). OMI is sensitive to metabolic behaviors caused by ER and HER2 (Fig. 3), known oncogenic drivers of glycolytic metabolism in breast cancer cells (3, 8, 37, 38). ER regulates gene expression of glucose transporter proteins and proteins involved in oxidative phosphorylation and the citric acid cycle such as isocitrate dehydrogenase, which actively reduces NAD^+ to NADH (37, 39–41). HER2 mediates metabolism through signaling of the PI3K/Akt/mTOR pathway, which directs transcription of glycolytic enzymes (2, 3). When combined, the redox ratio and NADH lifetime fully separate the distinct subtypes of breast cancer (Fig. 3D), indicating that the complementary measures of the redox ratio and fluorescence lifetime allow robust characterization of cellular metabolism and molecular microenvironments associated with breast cancer subtypes.

We further show that the OMI endpoints optical redox ratio, NADH τ_m , and FAD τ_m , reflect impaired metabolic activity in trastuzumab-responsive BT474 cells (Fig. 4) *in vitro*, suggesting a large metabolic response of HER2-amplified cells to trastuzumab. Only one of the OMI endpoints, NADH τ_m , decreased in the trastuzumab-resistant HR6 cells (Fig. 4). Although HR6 cells maintain HER2-overexpression and PTEN and P-Akt protein levels in the presence of trastuzumab, blockage of HER2 with trastuzumab may affect internal signaling (28). Because of its highly sensitive nature, NADH τ_m may be reporting such minute metabolism differences.

A subset of variables also resolved metabolic changes induced by trastuzumab in poorly responsive HER2-amplified cells (MDA-MB-361) *in vitro* (Fig. 4). Behavioral heterogeneity inherent to the MDA-MB-361 cell line suggests that 25% of the overall population would be growth inhibited by trastuzumab *in vitro* (42). Our data detected a 30% subpopulation

within MDA-MB-361 cells that responded to trastuzumab through decreased redox ratios (Fig. 4D), suggesting correlative evidence that high-resolution optical imaging is capable of detecting responders and nonresponders at the single-cell level in the context of a heterogeneous tumor cell population with a mixed response. The complementary metabolic information gained from the OMI endpoints allowed identification of a large metabolic response to trastuzumab (BT474) but also resolved negative (HR6) and partial (MDA-MB-361) responses, showing high sensitivity and resolution of OMI.

Finally, we measured the *in vivo* metabolic response to trastuzumab in HER2-overexpressing mouse xenografts. Trastuzumab-induced metabolic repression in BT474 and MDA-MB-361 tumors was detected by 48 hours posttreatment (Figs. 5 and 7). The redox ratio and FAD τ_m did not change in trastuzumab-resistant HR6 tumors treated with the antibody (Fig. 6). However, NADH τ_m decreased in trastuzumab-treated HR6 tumors compared with controls at 48 hours (Fig. 6), which was consistent with the *in vitro* results (Fig. 4). Modeling of the intercellular variation of MDA-MB-361 xenograft tumors identified two subpopulations in response to trastuzumab for the optical redox ratio and FAD τ_m (Fig. 7), suggesting *in vivo* cell-to-cell heterogeneity. This heterogeneous *in vivo* response is consistent with the *in vitro* results (in Fig. 4). We attempted to include only tumor cells in our image analysis, by evaluation of the cell morphology with respect to histology, but acknowledge that nontumor cell populations could have been included in this analysis. We speculate the greater intracellular variation of both the redox ratio and FAD τ_m observed in the MDA-MB-361 tumors treated with trastuzumab (Fig. 7H) is due to heterogeneous responses of individual mitochondria (43). Elucidation of this possibility will require additional research.

The tumor size measurements and IHC analysis of cellular proliferation and apoptosis were consistent with an antitumor effect of trastuzumab against BT474 tumors and a lack of an effect against HR6 tumors (Figs. 5 and 6). Tumor size measurements first identified a difference in control versus trastuzumab-treated BT474 tumor size at 8 days after treatment was initiated (Fig. 5A), 6 days later than the first significant change in OMI endpoints between control and trastuzumab-treated tumors (day 2, Fig. 5G). Although Ki67 staining of BT474 tumors identified reduced proliferation in BT474 tumors treated with trastuzumab at 2 and 5 days posttreatment, OMI is advantageous over IHC as a measure of tumor response because OMI provides a dynamic measure of cellular metabolism and can be conducted *in vivo* and over time within the same animal. Although *in vivo* OMI data are presented here, OMI endpoints remain robust measures of metabolism in freshly excised tissues (21) allowing implementation of this metabolic imaging technique in situations when *in vivo* measurements are not feasible.

FDG-PET has also been explored as a potential tool for assessing response to therapeutic agents. Consistent with a prior study (7), in this study, FDG-PET failed to identify a metabolic difference between control and trastuzumab-treated BT474 tumors (Fig. 5). FDG-uptake did increase over time as the control tumors continued to grow. Unlike OMI, PET cannot resolve cellular subpopulations of differing response

that are critical for investigating drug resistance mechanisms (7, 13–17, 44). Furthermore, PET requires large upfront and continued costs due to radiolabeled tracers, whereas OMI can be implemented in low-cost, wide-field, or confocal microscopes without the need for external dyes (45–47). In addition, OMI allows for fast, accurate, dynamic *in vivo* monitoring of early-therapeutic response, potentially reducing time and animals required for drug development. Our studies have focused on breast cancer and HER2 inhibition, but the methods developed are also applicable to the array of diseases that are treated with metabolism-modulating drugs (48–50).

The results of this study validate OMI as a powerful tool for quantifying cellular metabolism, which is an active area of investigation across multiple diseases. As we show using human breast cancer cells and xenografts, optical metabolic measurements are sensitive to distinct tumor cell subtypes. In addition, we present significant findings suggesting that OMI can be used as an early indicator of metabolic response to treatment with a targeted therapy, both in cell culture and *in vivo*. These results represent the culmination of multiple imaging technologies, analysis tools, and their validation with standard assays and FDG-PET. The results in breast cancer cells and xenografts provide the first direct measurements relating cellular metabolism, HER2 expression, HER2 inhibition, and resistance to HER2 inhibitors in live cells and tissues. In addition, these methodologies are potentially broadly applicable outside the cancer and imaging communities, including those in drug development and metabolism research across multiple diseases. Although we see OMI as an immediately powerful tool in preclinical models, it also may directly impact patient care as an adjunct to current practice, either on freshly

excised tissue (21) or through *in vivo* endoscopes adapted for fluorescence imaging.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.J. Walsh, H.C. Manning, M.C. Skala

Development of methodology: A.J. Walsh, R.S. Cook, M.C. Skala

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.J. Walsh, R.S. Cook, D.J. Hicks, A. Lafontant, M.C. Skala

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. Walsh, R.S. Cook, H.C. Manning, A. Lafontant, M.C. Skala

Writing, review, and/or revision of the manuscript: A.J. Walsh, R.S. Cook, H.C. Manning, A. Lafontant, C.L. Arteaga, M.C. Skala

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.C. Skala

Study supervision: M.C. Skala

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